PRIFYSGOL ABERYSTWYTH UNIVERSITY

Relationships between fatty acids, lipids and other characteristics of perennial ryegrass (*Lolium perenne*)

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A thesis submitted at the Institute of Biological, Environmental and Rural Sciences (Aberystwyth University) for the degree of Doctor of Philosophy

Declaration

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Summary

Production and consumption of ruminant products is partly being held accountable for the increasing global challenges of human health and climate change. Also, increasing demand for food, feed and fuel is placing growing pressure on land availability. One area under investigation in response to these challenges is fatty acid content of forages. This thesis sets out to investigate the variation and relationships between fatty acids, lipids, chlorophyll and other nutritional aspects of perennial ryegrass. Additionally, it will investigate alternative methods to predict fatty acids in forage. The core experiment involved twenty-four genotypes from two perennial ryegrass populations. Fatty acids were found to increase in leaf material during a growing season. Genotype differences in fatty acid content and composition were found which were broadly consistent across the growing season. Fatty acids correlated positively with crude protein but negatively with water-soluble carbohydrates. A positive and consistent relationship was found between chlorophyll and fatty acids across the growing season. The use of a chlorophyll meter to estimate fatty acid content did not perform very well, due to poor relationships with *in vitro* chlorophyll, however near-infrared reflectance and Fourier-transform mid-infrared spectroscopy had acceptable prediction accuracies for use as a screening tool. The accuracies of these prediction methods could be improved with further development using larger datasets. Investigation of the lipid composition revealed that galactolipid proportion was the main contributor to increased total fatty acid content in the high FA genotypes. While phospholipid proportion was minimally affected and neutral lipid negatively affected by increased total fatty acid content. Further work is needed to determine the underlying genetic control of fatty acid and lipid synthesis in perennial ryegrass. Additionally, a great deal more research is needed to establish environmental and genetic effects on lipid composition of forages.

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List of Abbreviations

1D-TLC	One-dimensional thin layer chromatography
2D-TLC	Two-dimensional thin layer chromatography
A_{645}	Absorbance at 645nm
A_{663}	Absorbance at 663nm
AA	Acetic acid
ACCase	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ADF	Acid detergent fibre
ADG	Average daily gain
ANOVA	Analysis of variance
ATR	Attenuated total reflectance
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1cis-9	Palmitoleic acid
C18:0	Stearic acid
C18:1cis-9	Oleic acid
C18:2n-6	Linoleic acid
C18:3n-3	A-Linolenic acid
C20:4n-6	Arachidonic acid
C20:5n-3	Eicosapentaenoic acid (EPA)
C22:5n-3	Docosapentaenoic acid (DPA)
C22:6n-3	Docosahexaenoic acid (DHA)
CFU	Colony forming unit
CH_4	Methane
$CHCl_3$	Chloroform
Chl	Chlorophyll
CLA	Conjugated linolenic acid
CO_2	Carbon dioxide
COOH	Carboxylic acid
CP	Crude protein

CV	Coefficient of variation
CVD	Cardiovascular disease
d.f.	Degrees of freedom
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DM	Dry matter
DMI	Dry matter intake
DOMD	Digestible organic matter in the dry matter
DT	Detrend
EE	Ether extract
ER	Endoplasmic reticulum
FA	Fatty acid
FAD	Fatty acid desaturase
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FDM	Freeze-dried matter
FFA	Free fatty acid
FTMIR	Fourier-transform mid-infrared
G3P	Glycerol-3-phosphate
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionisation detector
GHG	Greenhouse gas
GL	Galactolipid
HBV	High biological value
HCl	Hydrochloride
HPLC	High performance liquid chromatography
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
IVDMD	In vitro dry matter digestibility
KAS	Ketoacyl-ACP synthases
LAB	Lactic acid bacteria
LDL	Low density lipoprotein
MAG	Monoacylglycerol

ME	Metabolisable energy
MeOH	Methanol
MGDG	Monogalactosyldiacylglycerol
ML	Membrane lipid
MSEC	Mean standard error of calibration
MSECV	Mean standard error of cross-validation
MSEP	Mean standard error of prediction
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NDF	Neutral detergent fibre
NIR	Near-infrared reflectance
NL	Neutral lipid
PA	Phosphatidic acid
PAP	Phosphatidate phosphatase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PLS	Partial least squares
POL	Polar lipid
PPO	Polyphenol oxidase
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
r	Coefficient of determination
R2	Correlation coefficient
RPD	Ratio of performance to deviation
SD	Standard deviation
SEC	Standard error of calibration
SECV	Standard error of cross-validation
SEP	Standard error of prediction
SFA	Saturated fatty acid

SNV	Standard normal variate
SPAD	Soil plant analysis development
SPE	Solid phase extraction
SQDG	Sulfoquinovosyldiacylglycerol
TAG	Triacylglycerol
TFA	Total fatty acid
TL	Total lipid
TLC	Thin layer chromatography
UFA	Unsaturated fatty acid
UV	Ultraviolet
v/v	Volume to volume
w/v	Weight to volume
WSC	Water-soluble carbohydrate

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Chapter 1. Introduction

1.1 Introduction

There has been a great deal of scrutiny of late surrounding the production and consumption of ruminant products (Millward and Garnett, 2010). Firstly, there has been vast amounts of debate and discussion relating to the consequences consumption of ruminant products (meat and milk) may have on human health. Epidemiological studies carried out in the 1980's found positive associations between saturated fatty acid (SFA) intake and risk of cardiovascular disease (CVD), believed to be due to a rise in low-density lipoprotein (LDL) cholesterol (Luciano, 2009; Daley *et al.*, 2010). This resulted in the development of guidelines surrounding dietary fat intake which included specific recommended daily intakes for certain fat classes, such as SFA, *trans*-fatty acids, omega-3 (n-3) polyunsaturated fatty acids (PUFA) and omega-6 (n-6) PUFA (WHO, 2003). This was accompanied by recommendations to reduce red meat consumption due to its perceived high SFA content. Consequently, ruminant products (i.e. milk, beef and sheep meat) have not observed the same increase in consumption per capita compared to pig and poultry meat in developed countries (Kanerva, 2013). Progress in animal breeding and genetics has been successful in producing an overall leaner product (Higgs, 2000). Yet there remains a stigma surrounding red meat, with numerous publications relating red meat consumption with potential increased risk of non-communicable diseases such as CVD, diabetes and certain cancers.

In spite of this, red meat is an important source of high biological value (HBV) protein, vitamin B12, Iron and Zinc, amongst other important nutrients (Williams, 2007; McAfee *et al.*, 2010), and as such should not be completely excluded from the

diet. In addition, emphasis has been placed on the fact that red meat also contains health beneficial fats, including Vaccenic acid (C18:1*trans*-11), conjugated linoleic acids (CLAs) and n-3 PUFA. With the decreasing trend in the consumption of oily fish, the contribution of red meat to the total intake of these essential n-3 PUFA is becoming of increased importance (Russo, 2009).

A great deal of attention has been placed on dietary strategies to improve the fatty acid (FA) composition of ruminant products, with particular emphasis on increasing the beneficial fats (C18:1*trans*-11, CLAs and n-3 PUFAs). A variety of different dietary supplements have been investigated, including fish oils, seeds and seed oils, micro-algae and rumen protected lipid supplements (see reviews by Mir *et al.*, 2003; Raes *et al.*, 2004; Scollan *et al.*, 2006, 2011, 2014; Williams and Burdge, 2006; Sinclair, 2007; Woods and Fearon, 2009; Doreau *et al.*, 2011). However the basal forage diet of ruminants also has significant influence on the FA composition of meat and milk. With rising competition for land stemming from the 'food vs feed vs fuel' dispute (Spiertz and Ewert, 2009; Sunderasan, 2009; McNeill, 2012), more serious consideration is being given to the advantages of forage-fed ruminants. Indeed, there is now an expanding market for 'grass-fed' or 'grass-finished' ruminant products, fostered by the decreased total fat content and increased n-3 PUFA content of animals from forage-based relative to grain-based production systems (Daley *et al.*, 2010; Morgan *et al.*, 2012; Howes *et al.*, 2015). Accordingly, investigation into the FA content and composition of forages to further enhance the nutritional benefits of forage-fed ruminant products is gaining momentum. A number of studies have investigated management effects such as species and cultivar, cutting date (season), regrowth period, nitrogen fertilisation and conservation on FA content and

composition of forages (Dewhurst *et al.*, 2003; Glasser *et al.*, 2013); along with emerging interest in the genetic background of this trait (Hegarty *et al.*, 2013).

Another aspect which has received a great deal of attention lately is the environmental impacts of ruminant production, with particular concern over greenhouse gas emissions (methane and nitrous oxide) and water requirements (per kg beef produced). It was noted in 2006 that global livestock production accounted for approximately 9% of total anthropogenic carbon dioxide (CO_2) emissions. However this sector was responsible for approximately 37% of anthropogenic methane (CH_4) emissions, which has 23 times the global warming potential of CO_2 (Steinfeld *et al.*, 2006). More recently, Tubiello *et al.* (2013) showed that enteric CH_4 emissions accounted for 37% of total agricultural emissions at the end of 2010, and that non-dairy cattle were the primary animal type contributing to livestock green-house gas (GHG) emissions. This CH_4 is produced by methanogenic archaea as a result of inefficient fermentation from an imbalance of protein and energy availability within the rumen. Forage-based strategies to improve rumen efficiency and reduce CH_4 emissions have included improving fibre digestibility and increasing the energy density of forages through targeting increased water-soluble carbohydrate (WSC) content (Kingston-Smith *et al.*, 2010; Ellis *et al.*, 2012). It is predicted that targeting increased FA content will also increase the energy density of forages, leading to further improvements in rumen efficiency and in turn reduce CH_4 emissions (Barret *et al.*, 2014).

Thirdly, increasing the FA content of forages may open up the opportunity for the development of non-seed biomass oil crops, thus relieving some of the pressure on arable land for the production of biofuels/bioenergy (Winichayakul *et al.*, 2013; Vanhercke *et al.*, 2014).

The topics discussed here are of great relevance and consequence, in addition to a number of other issues not discussed including projected increased demand for animal-derived protein as developing countries become more affluent, issues surrounding animal welfare, increasing global population and urbanisation and food security (Godfray *et al.*, 2010a, 2010b). Developing forages with increased FA content and enhanced FA composition may offer the potential to alleviate, at least in part, the current and future challenges of human health, climate change and bio-energy.

1.2 Thesis Overview

This thesis focuses on advancing knowledge on fatty acids and lipids in perennial ryegrass. The research undertaken at the Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University involved a number of experiments, which are discussed in depth in the following chapters:

Chapter 2

Review of the literature relating to FA content and composition of forages with the purpose of introducing the topic and positioning the research in a wider context.

Chapter 3

Investigating the relationship between *in vivo* chlorophyll (estimated using a chlorophyll meter: *SPAD*-502) and FA content of perennial ryegrass to assess potential for *SPAD* to be used as a proxy for FA content of forage.

Chapter 4

Evaluating the effect variation in FA content may have on other nutritionally important characteristics of perennial ryegrass, particularly CP and WSC.

Chapter 5

Assessing the seasonal variability in FA content and composition of perennial ryegrass, along with investigation and validation of the inter-relationships between *in vivo* chlorophyll (*SPAD*-502), *in vitro* chlorophyll, FA content and FA composition.

Chapter 6

Establishing the relationships between content and proportions of FAs in perennial ryegrass across multiple-harvests during one growing season and appraising these relationships in view of current knowledge of FA and lipid biosynthesis in plants.

Chapter 7

Investigating how the lipid composition varies between perennial ryegrass genotypes that vary in total FA content and chlorophyll content, which included establishing and developing a new laboratory method.

Chapter 8

Assessing the potential use of NIR and FTMIR spectroscopy to predict FA content and composition of perennial ryegrass.

Chapter 9

Concludes the thesis by discussing the contribution of this research in the context of the overall topic while also discussing the limitations of the experiments and openings for future research directions.

1.3 Thesis Aims and Objectives

The aim of this thesis was to investigate the variation in and relationships between fatty acids, lipids and other constituents perennial ryegrass, along with prospective methods to predict these traits. A number of objectives were set out as follows:

- Assess the genotypic and seasonal variation in fatty acid and lipid content and composition of a perennial ryegrass
- Investigate the effect variation in fatty acid content has on other nutritional characteristics
- Quantify the relationships between fatty acids, lipids and other characteristics of perennial ryegrass
- Evaluate the potential of alternative methods to predict fatty acid content and composition

It is hypothesised that differences in fatty acid content and composition will be found between genotypes from the same population, and this variation in fatty acid content will have varying effects and relationships with other characteristics of perennial ryegrass across the growing season.

Chapter 2. Literature Review

2.1 Feeding Value of Forages

Grasses comprise mainly of energy-rich complex structural and non-structural carbohydrates (Humphreys, 2005). Due to the complexity of these carbohydrates, it is difficult to release this energy; however ruminants, as well as hindgut fermenters, are highly adapted to the digestion of this fibrous material through microbial fermentation (Hofmann, 1989; Mackie, 2002). Ruminants require adequate coarse, insoluble fibre in their diet to maintain healthy rumen function (Van Soest *et al.*, 1991). Fibre content of forages can be expressed in terms of neutral detergent fibre (NDF) and acid detergent fibre (ADF), from which the digestibility and metabolisable energy (ME) can be estimated. Forage is also a good source of protein, especially legumes (Humphreys, 2005), which is frequently expressed as crude protein (CP). Typical mean feed composition values of commonly used forages are presented in Table 2.1. A less frequently quoted forage composition value is ether extract (EE), which includes constituents such as lipids, FAs, waxes and sterols.

Interest in improving forages accelerated during the early 20th century due to the need for increased food production in the UK following the First World War (Casler and Vogel, 1999; Humphreys, 2005). A significant milestone during this period was the establishment of the Welsh Plant Breeding Station (WPBS) partnered with the introduction of laboratory analyses for crude fibre and protein concentrations, making the concept of breeding for increased forage quality more feasible (Casler and Vogel, 1999). Breeding traditionally focused on improving agronomic characteristics such as yield, persistency and disease resistance to increase output (Moorby *et al.*, 2008;

Table 2.1 Published feed composition values for dry matter (DM), crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), digestible organic matter in the dry matter (DOMD) and metabolisable energy (ME).

		Dry matter basis					
	DM ($g\ kg^{-1}$)	**CP** ($g\ kg^{-1}$)	**ADF** ($g\ kg^{-1}$)	**NDF** ($g\ kg^{-1}$)	**DOMD** ($g\ kg^{-1}$)	**ME** ($MJ\ kg^{-1}$)	**Reference**
Fresh grass, all species	197	156	296	577	710	11.2	MAFF (1992)
Grass silage	255	168	363	582	678	10.9	
Grass hay	865	107	367	657	596	8.8	
White clover (fresh)	118	298	253	400	699	11.6	
Maize silage	352	101	277	480	743	10.5	
Barley whole-crop silage	394	90	274	575	608	9.1	
Grass (Fresh)	180	160	300	620	-	11.3	Ewing (2002)
Grass silage	240	137	360	540	-	11.0	
Grass Hay	870	104	380	688	-	8.5	
Maize silage	300	90	300	550	-	11.5	
Straw	870	40	510	844	-	6.5	
Whole crop silage	400	95	350	540	-	10.5	
Grass, young (75-80D)	200	156	289	572	-	12.2	McDonald *et al.* (2011)
Grass, mature (60-65D)	282	100	312	647	-	10.0	
Grass silage, young	250	186	359	566	-	11.6	
Grass silage, mature	294	125	387	603	-	10.0	
Grass hay, poor-quality	800	55	452	725	-	7.0	
Grass hay, good-quality	900	110	364	650	-	9.5	
Red clover (fresh)	190	179	-	272	-	10.2	
White clover (fresh)	190	237	253	400	-	9.0	
Maize whole crop silage	210	110	277	480	-	10.8	

Wilkins and Humphreys, 2003). Focus then included improving characteristics such as digestibility and dry matter intake (DMI), accompanied by the recognition that grass is a key factor in production, economic and sustainability issues of livestock (Sampoux *et al.*, 2011). Casler and Vogel (1999) stated that a 1% increase in *in vitro* dry matter digestibility (IVDMD) resulted, on average, in a 3.2% increase in average daily gains (ADG) of beef cattle. Digestibility can be improved by either increasing the digestibility of the fibre (mainly cell walls) or improving the ratio between fibre and cell contents, such as CP or WSC (Wilkins and Humphreys, 2003). Attention was also given to improving efficiency through better nitrogen use efficiency and extending the growing season (Moorby *et al.*, 2008; Humphreys, 2005). This has led to the development of distinctive grass varieties, such as high-sugar grasses which have been shown to aid factors such as protein use, voluntary feed intake and potentially reduce methane emissions, although this is still under debate (Ellis *et al.*, 2012). Future breeding targets will need to consider issues such as consumer demands for enhanced quality and functionality of products, reducing environmental impacts of ruminant products and finding alternative uses for forages such as biofuel production. Combining traditional forage breeding methods with new technologies such as genetic fingerprinting and marker-assisted selection will aid in the development of new and novel forage varieties (Humphreys, 2005; Kingston-Smith and Thomas, 2003).

One area currently under investigation to address the above issues is the FA content and composition of forages. Although forage has a low fat content, it can contribute greatly, and in some cases wholly, to the total amount of fat ingested by ruminants due to the large amount of dietary forage material consumed (Dewhurst *et al.*, 2003; Hawke, 1973). For instance, dairy cows can consume between 15 and 20kg DM d^{-1} meaning that total fat intake may be up to 500g d^{-1} from forage alone (Elgersma *et al.*,

2003a). It is hoped that enhancing the FA content and composition of forages will offer the opportunity to 1) improve the FA composition of ruminant products (i.e. meat and milk), 2) increase the energy density of forages to improve the efficiency of ruminants, and 3) provide alternative, non-seed biomass oil crops. A number of studies have highlighted a strong genetic correlation with regard to FA content and composition, suggesting there is potential to breed for higher fat content (Dewhurst *et al.*, 2001). Furthermore, Hegarty *et al.*, (2013) have successfully identified regions of the perennial ryegrass (*Lolium perenne*) genome which are associated with FA content. However, large genotype x environment interactions have also been identified (Dewhurst *et al.*, 2003; Palladino *et al.*, 2009). Understanding, monitoring and manipulating these genetic and environmental factors will be key in the exploitation of high lipid grasses (Kingston-Smith and Thomas, 2003; Dewhurst *et al.*, 2001; Kingston-Smith *et al.*, 2010).

2.2 Chemical Structure and Nomenclature

2.2.1 Fatty Acids

Fatty acids are usually unbranched, even numbered hydrocarbon chains with a terminal carboxylic acid (COOH) group. Chain length can vary from anything up to 24 carbons, but usually contain 16 or 18 (Hames, 2005). They can be referred to either by their trivial name, their systematic name according to the International Union of Pure and Applied Chemistry (IUPAC) or by shorthand abbreviations, as shown in Table 2.2 for some common FAs. Concerning the IUPAC nomenclature method which gives rise to the systematic names (IUPAC, 1978), numbering of Carbon atoms begins at the carboxyl end, with the carboxyl Carbon itself referred to as C1, the one next to that C2 and so forth. Greek letters can also be assigned to Carbon atoms. The Carbon

atom next to the carboxyl group (C2) is considered alpha (α), next to that is beta (β), then gamma (γ) and so on in the direction of the methyl end. However, the final carbon is always known as omega (ω), which can also be denoted by the letter *n*.

Table 2.2 Trivial, Systematic and shorthand abbreviation names for some common fatty acids

Trivial name	Systematic name	Shorthand abbreviation
Lauric acid	Dodecanoic acid	C12:0
Myristic acid	Tetradecanoic acid	C14:0
Palmitic acid	Hexadecanoic acid	C16:0
Palmitoleic acid	Hexadec-9-enoic acid	C16:1*cis*-9
Stearic acid	Octadecanoic acid	C18:0
Oleic acid	Octadec-9-enoic acid	C18:1*cis*-9
Linoleic acid	9,12-Octadecadienoic acid	C18:2n-6
α-Linolenic acid	9,12,15-Octadecatrienoic acid	C18:3n-3
Arachidonic acid	5,8,11,14-Eicosatetraenoic acid	C20:4n-6
Timnodonic acid	5,8,11,14,17-Ecosapentaenoic acid	C20:5n-3
Clupanodonic acid	7,10,13,16,19- docosapentaenoic acid	C22:5n-3
Cervonic acid	4,7,10,13,16,19-docosahexaenoic acid	C22:6n-3

Fatty acids can be categorised into a number of different groups, depending on the nature of the FA. Saturated fatty acids (SFA) contain no C=C double bonds; an example of which is Palmitic acid (C16:0) which is the most widely occurring SFA (Gunstone, 1999). Unsaturated FAs, however, contain one or more C=C double bonds. Unsaturation is most prevalent in FAs with either 18 or 20 carbon atoms (Smith, 1991). Unsaturated FAs can be further categorised into mono-unsaturated fatty acids (MUFA) which have only one C=C double bond, such as Oleic acid (C18:1*cis*-9); or poly-unsaturated fatty acids (PUFA) which have more than one C=C double bond, such as α-Linolenic acid (C18:3n-3). The presence of these double bonds means that different geometrical isomers exist, depending on the position of the atoms either side of the double bond. The two isomers that do exist are shown in Figure 2.1. A *cis* configuration is when the atoms are on the same side whereas a *trans* configuration is

when the atoms are on opposite sides. The structural differences between SFA, MUFA and PUFA are shown in Figure 2.2 exemplifying an 18C FA.

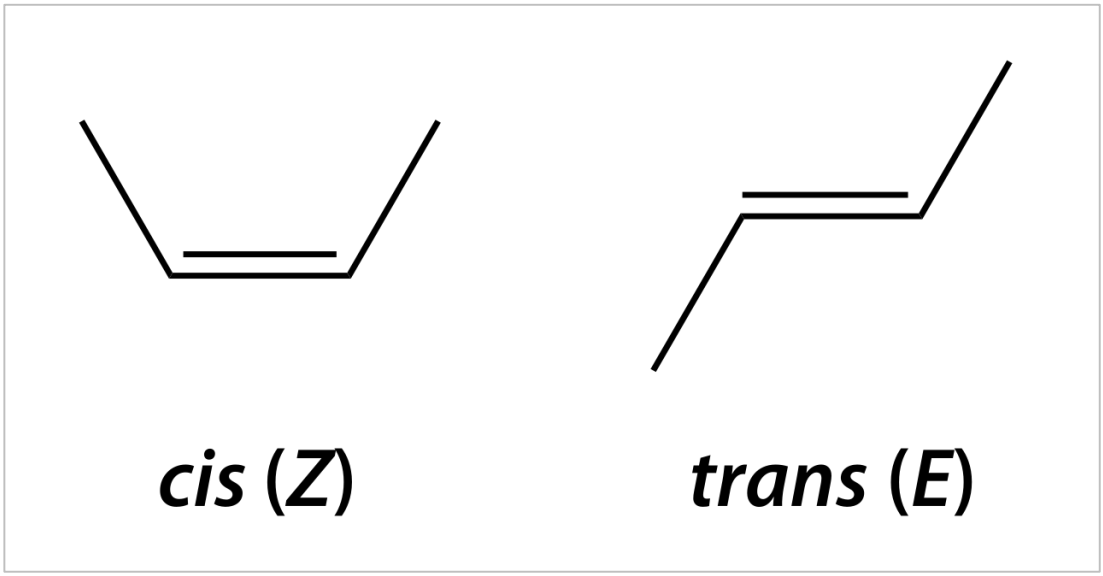

Figure 2.1 Schematic illustrating a *cis* versus a *trans* double bond configuration

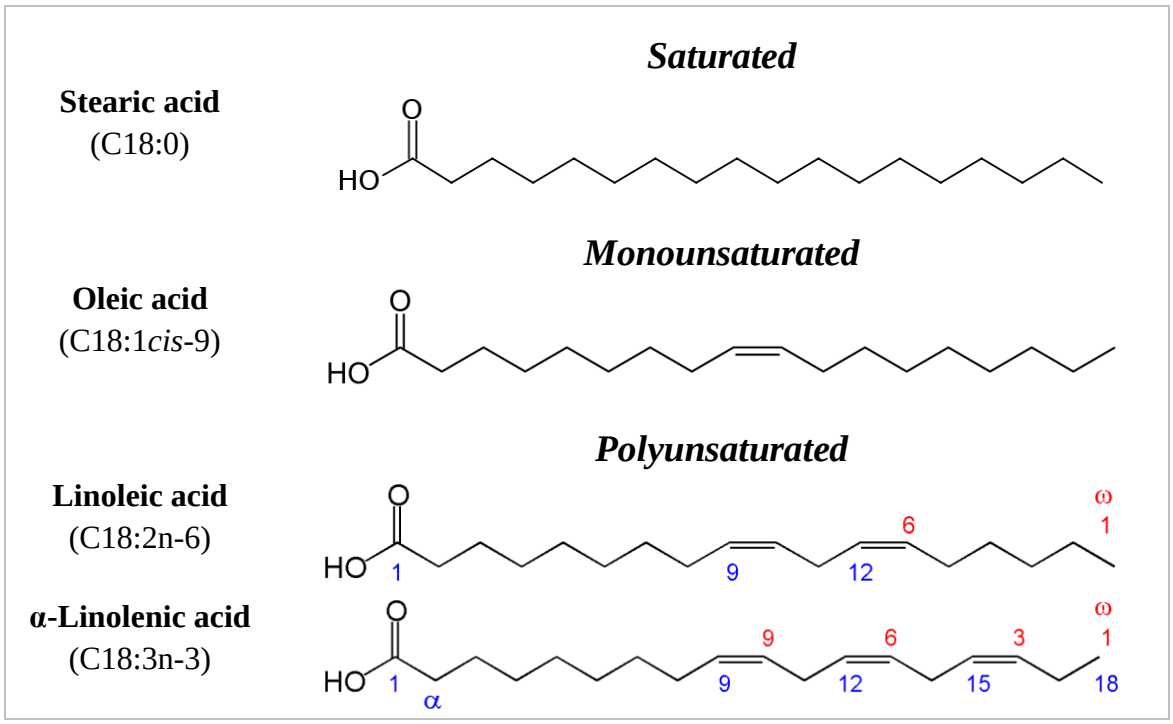

Figure 2.2 Schematic showing the differences in structure between saturates, monounsaturated and polyunsaturated fatty acids

2.2.2 Lipids

Fatty acids rarely occur in their free form and are more frequently found in lipid form as esters, which contain alcohols such as glycerol, or occasionally as amides (Gunstone, 1999). The term ‘lipid’ can encompass a varying number of compounds depending on the definition used, as there is at present a lack of a widely accepted definition of the term. The majority of textbooks would generalise lipids as compounds that are soluble in organic solvents such as chloroform or alcohol. However a more explicit definition of the term is “fatty acids and their derivatives, and substances related biosynthetically and functionally to these compounds” (Christie, 2003). They can largely be split into two groups, namely the ‘neutral’ or ‘simple’ lipids and the ‘polar’ or ‘complex’ lipids. ‘Neutral’ lipids generally include free fatty acids (FFA), acylglycerols and some ether lipids, sterols and waxes. ‘Polar’ lipids, on the other hand, include glycolipids, phospholipids and sphingolipids.

Focusing on the structure of glycerolipids, these consist of various FAs and head groups esterified to the hydroxyl groups of glycerol. Acylglycerols consist of glycerol and FAs only and can exist in mono-, di- or tri- acylglycerols, depending on the number of FAs esterified to the glycerol back bone. Glycolipids contain a sugar head group in addition to the glycerol backbone and FAs, whereas phospholipids include phosphate as the head group. The differences in chemical structure between triacylglycerol, galactolipid and phospholipid are shown in Figure 2.3. Concerning the nomenclature of lipids, they are most commonly referred to by their trivial names or by shorthand abbreviations, examples of some common glycerolipids are given in Table 2.3. They are also granted systematic names, as governed by the IUPAC (1978).

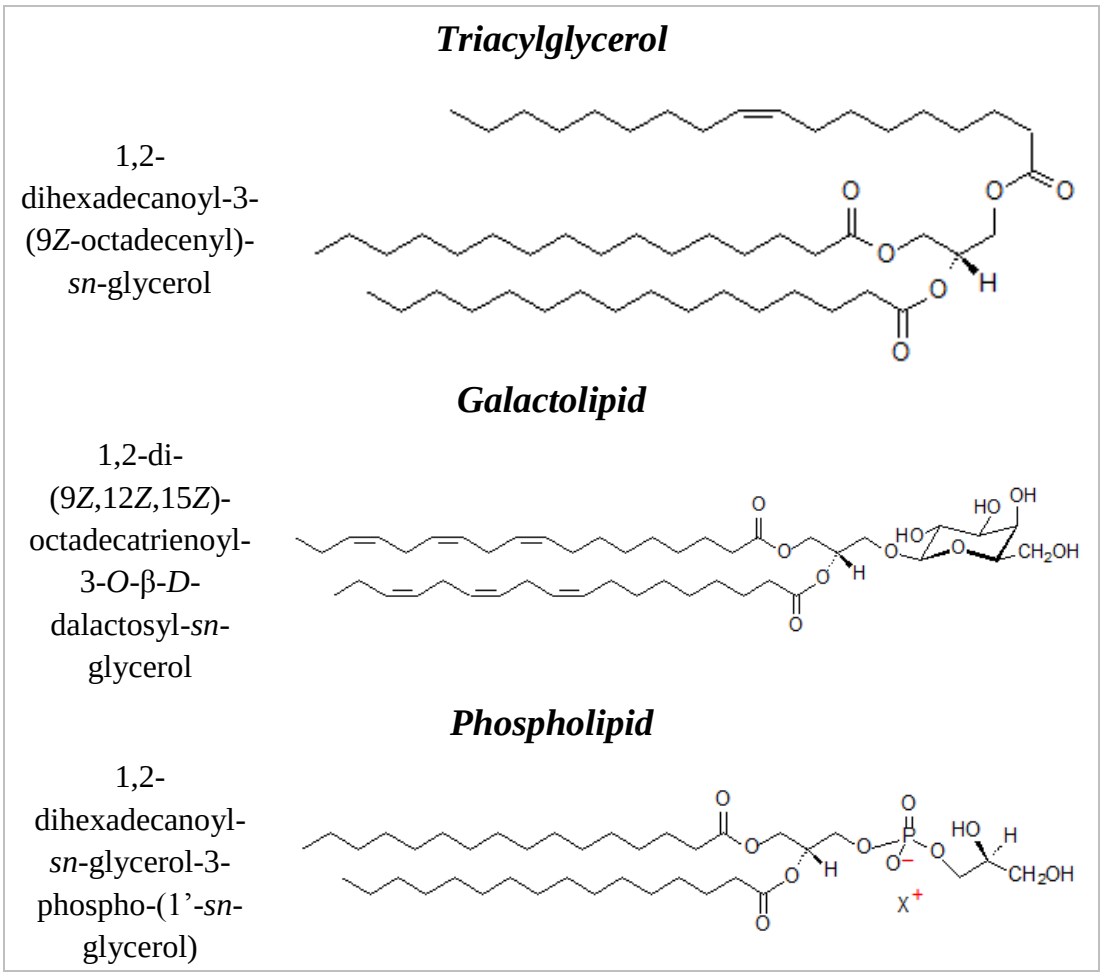

Figure 2.3 Chemical structures of triacylglycerol, galactolipid and phospholipid

Table 2.3 Trivial names and shorthand abbreviations for some common glycolipids and phospholipids

Trivial name	Shorthand abbreviation
Acylglycerols	
Triacylglycerol	TAG
Diacylglycerol	DAG
Monoacylglycerol	MAG
Glycolipids	
Monogalactosyldiacylglycerol	MGDG
Digalactosyldiacylglycerol	DGDG
Sulfoquinovosyldiacylglycerol	SQDG
Phospholipids	
Phosphatidic acid	PA
Phosphatidylcholine	PC
Phosphatidylglycerol	PG
Phosphatidylethanolamine	PE
Phosphatidylinositol	PI
Phosphatidylserine	PS

2.3 Plant Lipid Biochemistry

Plants produce the majority of the world's lipids, which function mainly as the basic components of cellular membranes, as an important store of energy and play a role in acute biological activities (Harwood, 1996). Additionally in plants, they function as constituents of the plant surface layers. Plants are also the primary source of n-6 and n-3 FAs in both terrestrial and marine ecosystems; as they have the unique ability to synthesise *de novo* C18:2n-6 and C18:3n-3 which are the building blocks for the n-6 and n-3 series of long-chain essential FAs, respectively, via elongation and desaturation pathways (Dewhurst *et al.*, 2006, 2003; Barceló-Coblijn and Murphy, 2009).

2.3.1 Fatty Acid Synthesis

The primary location of FA synthesis in plants is within the plastid; and involves the cyclic condensation of two-carbon units to a precursor molecule Acetyl-CoA (Taiz and Zeiger, 2007). A series of enzymes are involved in synthesising FAs which are thought to be held together in a complex or metabolon referred to as Fatty Acid Synthase (FAS). Figure 2.4 illustrates the steps involved in the cycle of FA synthesis. The first committed step of FA synthesis is the formation of Malonyl-CoA from Acetyl-CoA and bicarbonate, via the enzyme Acetyl-CoA carboxylase (ACCase) (Sasaki *et al.*, 1995). Tight regulation of this enzyme appears to control the overall rate of FAS (Harwood, 1996; Ohlrogge and Jaworski, 1997). Indeed, Page *et al.* (1994) demonstrated that ACCase controlled 45-61% of flux to lipids in Barley and Maize. The Malonyl-CoA is then transferred to an acyl carrier protein (ACP) to form Malonyl-ACP. From here, 2C units are added via a series of condensing enzymes names β-ketoacyl-ACP synthases (KAS). The first condensing enzyme used is KASIII, which condenses Malonyl-ACP with Acetyl-CoA to form a 4C keto-intermediate,

Acetoacetyl-ACP (Harwood, 2005; Taiz and Zeiger, 2007). This keto-intermediate then undergoes a reduction, dehydration and a second reduction to produce Butyryl-ACP, a 4C FA attached to ACP. Further condensation reactions are catalysed by KASI, which produces 6-16C FAs, while KASII is used for the final condensation of C16:0 to C18:0. Termination of FAS sometimes occurs at the C16:0-ACP stage; however the majority of molecules are elongated to 18:0-ACP which is then efficiently desaturated to C18:1-ACP (most commonly via stearoyl-ACP Δ9-desaturase). These acyl-FAs are released from ACP via thioesterase enzymes to yield Palmitate (C16:0) and Oleate (C18:1), which are then converted to acyl-CoAs for extra-plastidal lipid assembly (Harwood, 2005).

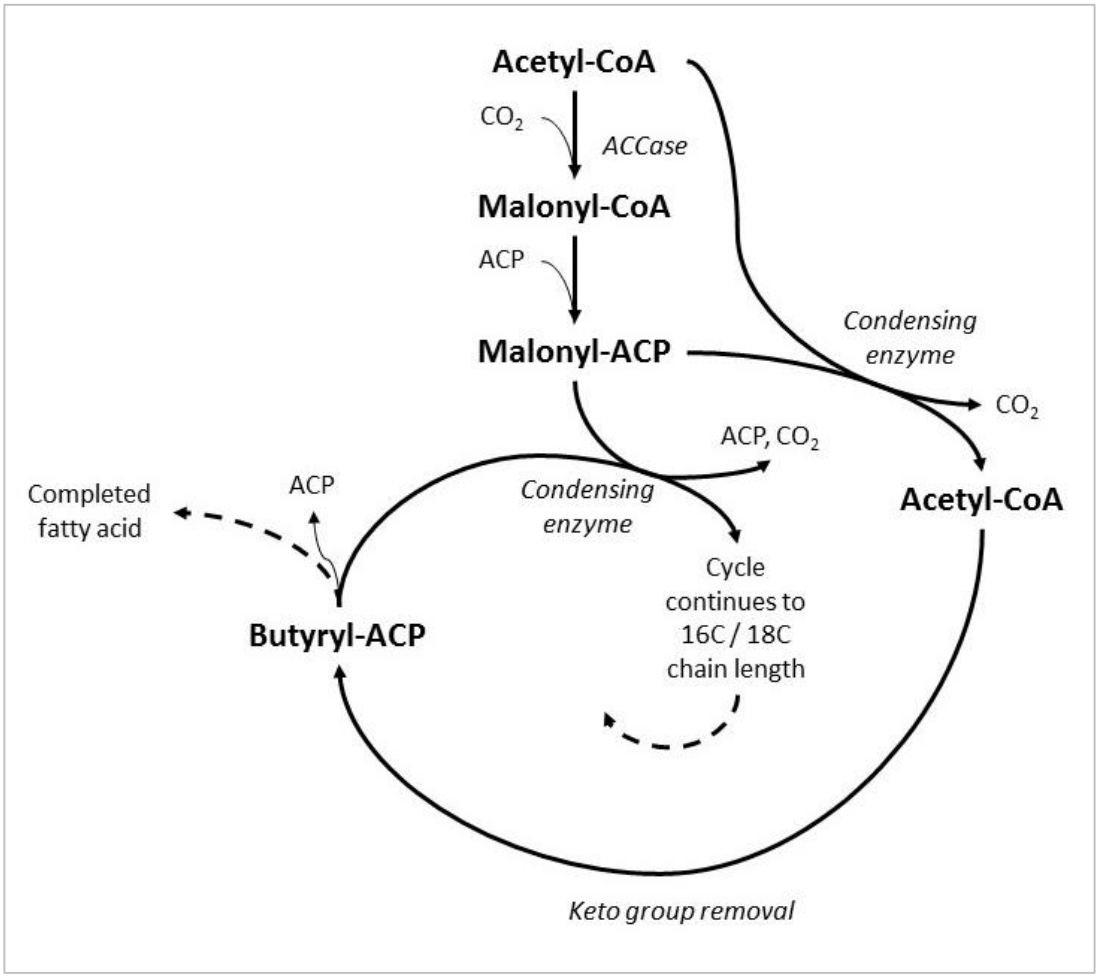

Figure 2.4 Fatty acid synthesis cycle in plants (adapted from Taiz and Zeiger, 2007)

2.3.2 Lipid Synthesis

There are two pathways to lipid synthesis in plants, namely the 'prokaryotic' and the 'eukaryotic' pathways, which are illustrated in Figure 2.5. The former applies to lipid synthesis within the chloroplast while the latter relates to lipid synthesis via the endoplasmic reticulum (ER) with subsequent modification of lipids in the plastid (Buchanan *et al.*, 2007). Plants that are capable of lipid synthesis via both pathways are deemed 'C16:3n-3' plants, due to the presence of C16:3n-3 in monogalactosyldiacylglycerol (MGDG; Dormann, 2005). Examples of 'C16:3n-3' plants include members of the *Apiaceae* (e.g. parsley) and *Brassicaceae* (e.g. rapeseed) families (Heinz and Roughan, 1983; Mongrand *et al.*, 1998a). However, members of the *Poaceae* family (e.g. perennial ryegrass) are deemed 'C18:3n-3' plants, due to the absence of C16:3n-3 from the 'prokaryotic' lipid synthesis pathway.

Focusing on the 'eukaryotic' pathway of lipid synthesis, final products of FAS are released from the chloroplast and transported to the ER in the form of acyl-CoA, where they are incorporated with glycerol-3-phosphate (G3P) to produce phosphatidic acid (PA). The ER derived PA gives rise to other phospholipids such as PC, PG, PE, PI and PS. The vast majority of PC is then transferred back to the chloroplast, and the diacylglycerol moiety of this lipid is used to produce the galactolipids and sulfolipids (Mongrand *et al.*, 1998; Buchanan *et al.*, 2007).

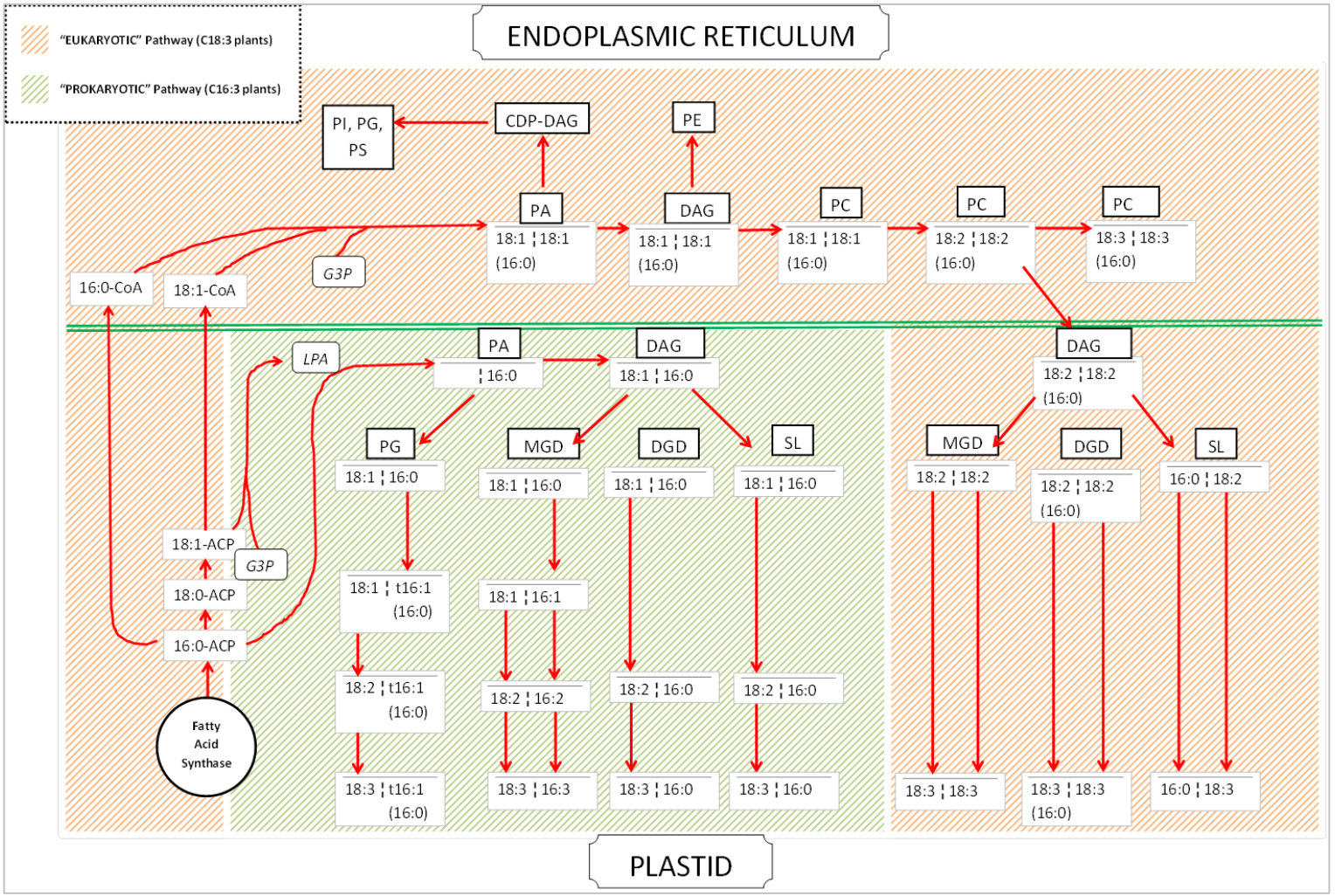

Figure 2.5 Schematic of the 'prokaryotic' and 'eukaryotic' lipid synthesis pathways of C18:3n-3 and C16:3n-3 plants (adapted from Harwood *et al* (1996)

2.4 Factors Affecting Forage Fatty Acids

Fatty acid content and composition of forage has been shown to be affected by species, regrowth interval, leaf to stem ratio, season, growth stage and maturity, fertilizer regime, temperature and light intensity (Palladino *et al.*, 2009; Hawke, 1973). Some of these factors are briefly discussed in more detail below; however they are also discussed in a recent review by Glasser *et al.* (2013).

2.4.1 Species, Cultivar and Genetics

Species differences in terms of FA content and composition have been reported by Dewhurst *et al.* (2001) and Boufaïed *et al.* (2003). In the case of Dewhurst *et al.* (2001), they reported that FA profiles were distinct between species when compared under the same cut/management yet this distinction was less clear when species were compared across cuts. This may be explained by the highly significant genotype x cutting date interactions ($P<0.001$). Table 2.4 presents typical FA content of various grass and legume species.

Differences between cultivars is less consistent, with no variation found by Dewhurst *et al.* (2002) and no variation in terms of TFA but variation found in proportion of C18:3n-3 by Gilliland *et al.* (2002) and Elgersma *et al.* (2003b). It should be noted that these studies used a small number of cultivars which may have had low genetic variability (Palladino *et al.*, 2009).

Table 2.4 Fatty acid content of selected forage species averaged across cultivars (adapted from Boufaïed *et al.*, 2003)

		Fatty acids (g kg^{-1} DM)					
		C16:0	**C18:0**	**C18:1 *cis*-9**	**C18:2 n-6**	**C18:3 n-3**	**Total**
Grasses	*Annual ryegrass*	4.71	0.45	1.12	3.99	17.89	29.14
	Meadow fescue	4.18	0.30	1.60	3.43	11.96	22.30
	Cocksfoot	3.94	0.38	0.78	3.73	11.54	21.07
	Smooth bromegrass	3.43	0.26	0.60	3.25	9.94	18.09
	Timothy	3.52	0.37	1.11	3.83	8.45	17.96
Legumes	*White clover*	4.98	0.83	1.45	4.64	16.04	28.95
	Red clover	4.06	0.81	1.73	4.99	9.24	21.54
	Lucerne	4.09	0.76	1.01	3.75	6.55	16.77

2.4.2 Season and Stage of Maturity

The effect of season and stage of maturity on lipid content and composition appears to be coherent between authors. High concentrations of FA occur during primary growth, leafy regrowth and vegetative growth late in the season (Hawke, 1973; Bauchart *et al.*, 1984; Dewhurst *et al.*, 2001). Bauchart *et al.* (1984) found the lipid content of perennial ryegrass to be highest during early May and late September (spring and autumn) and at its lowest at the beginning of August (summer). This pattern has also been found with hybrid and Italian ryegrass by Dewhurst *et al.* (2001), however Elgersma *et al.* (2003a) found the highest concentrations of FAs during the summer. Boufaïed *et al.* (2003) and Palladino *et al.* (2009) reported that season significantly affected TFA, C16:0, C16:1, C18:2n-6 and C18:3n-3 but not C12:0, C14:0, C18:0 or C18:1*cis*-9.

Leaf to stem ratio is an important factor in relation to FA content and composition, with 18:3n-3 proportion of young leafy grass ranging between 0.74 and 0.79 and older grass between 0.59 and 0.68 for perennial ryegrass harvested in autumn (Hawke, 1973). Fatty acid content declines from early to mid-season in line with reproductive

stem growth, then increases again towards the end of the growth season as leaf content recovers. This effect of leaf proportion may explain the inconsistent results found by Elgersma *et al.* (2003a) as the cuts taken in the summer months were from leafy regrowths whereas spring cuts contained 'stemmy' regrowths, hence this cut resulted in the lowest FA concentrations. Dewhurst *et al.* (2003) noted that Italian ryegrass remains 'stemmier' for longer than perennial ryegrass with hybrid ryegrass being intermediate.

2.4.3 Cutting and Regrowth Interval

Extended regrowth intervals have a negative impact on TFA and FA concentration (Elgersma *et al.*, 2005). Dewhurst *et al.* (2001) found FA losses of 17%, 25%, 34% and 45% for C18:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3, respectively, between 20d and 38d regrowth interval. In contrast to this, Elgersma *et al.* (2003b) found all FA decreased with increased regrowth interval, apart from C18:0, C18:1*cis*-9 and C18:2n-6. They also reported lower loss of C18:3n-3 (15-23%); however this may be explained by the smaller difference in regrowth interval of 10d in their study compared to 18d in the study of Dewhurst *et al.* (2001). Elgersma *et al.* (2005) also reported the effects of regrowth interval on FA proportions and found that the proportion of C16:1 and C18:3n-3 decreased while C16:0, C18:1*cis*-9 and C18:2n-6 increased with a longer regrowth period. It seems that management which inhibits flowering can increase FA content, as in the case of Bauchart *et al.* (1984) with two early cuts and Dewhurst *et al.* (2002) with nine cuts per year.

2.4.4 Fertiliser Regime

The few recent studies which have investigated the effect of Nitrogen (N) on FA content and composition report a positive effect of N fertilization on FA concentration (Boufaïed *et al.*, 2003; Elgersma *et al.*, 2005; Witkowska *et al.*, 2008; Salcedo, 2011). Boufaïed *et al.* (2003) found N fertilization increased TFA as well as the concentrations of C14:0, C16:0, C16:1, C18:1*cis*-9, C18:2n-6 and C18:3n-3 but had no effect on C12:0 and C18:0. Elgersma *et al.* (2005) and Salcedo (2011) also reported increases in TFA, C16:0, C16:1, C18:1*cis*-9, C18:2n-6 and C18:3n-3 with N fertilization, with no significant effect of proportions of individual FAs. Interestingly, the largest response to N fertilization in the study of Salcedo (2011) was with the intermediate treatment of 12 kg N ha^{-1} $month^{-1}$ under grazing conditions, with increases of 7.5%, 19%, 10%, 7.3% and 23% of C16:0, C18:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3, respectively, when compared to the 0 kg N ha^{-1} $month^{-1}$ treatment. Boufaïed *et al.* (2003) observed increases of 18%, 12% and 40% of C16:0, C18:2n-6 and C18:3n-3, respectively, with an overall increase of 26% TFA when comparing 120 vs. 0 kg N ha^{-1}. This effect was consistent across a wide range of vegetative stages and contrasting environmental conditions in the study by Witkowska *et al.* (2008).

Elgersma *et al.* (2005) noted a strong positive linear relationship between actual CP concentration and C18:3n-3 concentration, in line with Kemp *et al.* (1965) and Boufaied *et al.* (2003). There is no N contained within FA therefore the relationship between N fertilization and FA concentration must be indirect. It is hypothesized that higher N availability stimulates grass DM production, therefore increasing leaf area and stimulating synthesis of metabolic compounds, including chlorophyll which is positively correlated with chloroplast lipid, and thus increasing FA concentration (Witkowska *et al.*, 2008; Hawke, 1973). However further investigation is needed to

understand this interaction and also whether N has a direct effect on FA metabolism (Elgersma *et al.*, 2005; Witkowska *et al.*, 2008).

2.4.5 Conservation Method

Pasture is richer in FA, particularly C18:3n-3, when compared to conserved forage, as demonstrated by French *et al.* (2000) who reported that although the grass and grass silage used in their study had similar FA profiles, grass contained more unsaturated and less saturated FAs than silage. The extent of PUFA loss is dependent on method of conservation (Harfoot and Hazlewood, 1997). The main factors which may be responsible for FA losses during conservation are microbial intervention during ensiling causing undesirable fermentations, oxidation during field wilting and lipolysis via primarily plant lipases (Lough and Anderson, 1973; Dewhurst and King, 1998; Dawson *et al.*, 1977; Dewhurst *et al.*, 2003). These lipases are released in response to stress or natural senescence and cause the rapid release and degradation of membrane FAs, mainly C18:3n-3 and C18:2n-6.

The majority of research investigating FA changes during forage conservation suggests that the main period where FA loss is most likely to occur is during field wilting. Boufaïed *et al.* (2003) found lower concentrations of all the major UFA's (C16:1, C18:1*cis*-9, C18:2n-6 and C18:3n-3) and TFA in wilted grass compared to fresh grass. Elgersma *et al.* (2003b) and Dewhurst and King (1998) found comparable results, with marked decrease in TFA and proportion of C18:3n-3, especially under extended wilt conditions (Dewhurst and King, 1998). Conversely Arvidsson *et al.* (2009) noted that wilting did not affect FA proportions; however this difference may be due to differences in duration of wilting and/or differences in developmental stage. Interestingly, Chow *et al.* (2004) investigated changes in FA content and composition during wilting and ensiling using 3 different cultivars of perennial ryegrass, namely

Agri, Respect and Barnham. They found that wilting and ensiling had a comparable effect on C18:3n-3 proportion to that reported by Elgersma *et al.* (2003b) in two of the cultivars but no major effect on FA composition in Barnham cultivar.

Findings on the effect of additives on FA content and composition conflict somewhat. Arvidsson *et al.* (2009) reported that additives had an effect on fermentation characteristics but did not affect FA concentrations to a great extent. Dewhurst and King (1998) reported significant but relatively minor effects on levels and proportions on FA in perennial ryegrass silage. Elgersma *et al.* (2003b) also found that the use of additives also had minor effects, whereas Boufaïed *et al.* (2003) reported greater additive effects in silage and haylage from timothy. In their study, a lactic acid bacteria (LAB) additive lowered TFA and C18:3n-3 concentrations, formic acid decreased C16:0, C18:2n-6, C18:3n-3 and TFA concentration in silage and haylage and formalin lowered the proportions of C18:2n-6, C18:3n-3 and TFA. The dose rate of both LAB and formic acid generally had no significant effect on FA concentrations, apart from C18:2n-6 which was lower at the higher LAB dose rate of 10^5 CFU^x g^{-1} of FM compared to 10^6 CFU^x g^{-1} of FM.

2.5 Factors Affecting Lipid Composition of Forages

As mentioned earlier in this chapter, the three major lipids found in plants are the acylglycerols, phospholipids and galactolipids; broadly functioning as storage lipid, plasma membrane and chloroplast (thylakoid) membrane, respectively. The main phospholipids found in plasma membranes are PC and PE along with smaller amounts of PI and PS. While the major galactolipids are MGDG and DGDG, with smaller amounts of SQDG. Table 2.5 adapted from Guschina *et al.* (2014) shows the typical proportions of these lipids in various plant tissues.

Table 2.5 Typical lipid composition of different plant tissues from *Arabidopsis thaliana* (adapted from Guschina *et al.*, 2014)

Plant tissue		Lipid						
		PC	**PE**	**PI**	**PG**	**MGDG**	**DGDG**	**SQDG**
Leaf		10	5	3	8	40	28	6
Mitochondria	*Outer*	68	24	5	2	-	-	-
	Inner	29	50	2	1	-	-	-
Plasma membrane		32	46	19	tr.	-	-	-
Thylakoid		2	tr.	-	10	48	31	8
Root		35	28	14	-	-	-	-

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyl-diacylglycerol; SQDG, sulfoquinovosyldiacylglycerol

Studies that have investigated lipid composition of forages are much more limited compared to forage FA studies, with many dating back to the 1960s and 1970s. The majority of the work which has been carried out has been to better understand FA and lipid synthesis in plants, with the vast majority of these using C16:3n-3 plants such as *Arabidopsis thaliana* or spinach. Nevertheless, some factors which have been investigated in relation to lipid composition of forages are discussed below.

2.5.1 Stage of Maturity

Early work by Hudson and Karis (1974) investigated the effect of maturity on leaf lipids in ryegrass, kale and fodder radish, with specific attention given to fodder radish in terms of lipid composition (Table 2.6). They found that galactolipids are fairly consistent during the young, vegetative stage (12 days) through to the mature stage (56 days), then start to decline towards the senescent stage (100 days). Phospholipids also decrease with increasing maturity whereas an increase was observed in the non-polar lipids, particularly hydrocarbons, steryl esters, TAG and FFA.

Table 2.6 Lipid composition of fodder radish at different stages of maturity (adapted from Hudson and Karis, 1974)

	Crop maturity (days after sowing)					
Lipid class	**12**	**28**	**56**	**84**	**100**	**Average**
MGDG	25.0	19.6	16.8	17.0	8.7	17.4
DGDG	16.6	17.9	19.2	12.5	9.2	15.1
SQDG	10.1	10.9	13.0	11.8	5.6	10.3
TGDG (?)	1.1	2.8	0.9	0.8	0.3	1.2
Galactolipids	*52.8*	*51.2*	*49.9*	*42.1*	*23.8*	*44.0*
PE	9.3	8.3	7.1	6.5	4.8	7.2
PG	6.5	5.4	3.1	4.7	8.3	5.6
PC	5.6	3.6	2.4	1.6	2.4	3.1
PI	1.6	1.7	1.2	1.1	0.7	1.3
Phospholipids	*23.0*	*19.0*	*13.8*	*13.9*	*16.2*	*17.2*
pigments/non-polar	21.5	23.0	22.8	24.5	34.1	25.2
SG	2.7	6.4	13.5	19.4	25.9	13.6
other	*24.2*	*29.4*	*36.3*	*43.9*	*60.0*	*38.8*

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; SG, steryl glycosides

2.5.2 Wilting and Ensiling

The majority of studies involving lipid fractionation as part of their investigations into wilting and ensiling effects have been primarily focussed on endogenous plant factors such as polyphenol oxidase (PPO). Comparatively recent work by Lee *et al.* (2006) reported the lipid composition of different silage types in terms of polar lipids (POL), DAG, TAG and FFA (see Table 2.7). They found silages that contained red clover had higher POL proportions but conversely had a lower proportion of TAG. This work also showed the effect forage conservation has on the lipid composition, resulting in a much higher proportion of FFA, relative to what would typically be seen in fresh forage. Van Ranst *et al.* (2009) also investigated the effects of ensiling on lipid metabolism using ryegrass, red clover and white clover silages. They also reported an increase in FFA and a decrease in membrane lipids (ML). This increase in FFA is due to lipolysis via plant lipases activated due to stress, in addition to microbial lipases.

Table 2.7 Lipid composition of different silage types (adapted from Lee *et al.*, 2006)

Lipid fraction	Silage type					
	HSG	C	HSG and RC	C and RC	RC	Sig.
POL	23.6[c]	19.9[d]	28.0[a]	24.2[b]	28.4[a]	0.019
DAG	9.2	8.2	8.7	8.0	9.8	NS
TAG	24.4[b]	26.5[a]	19.2[c]	19.0[c]	15.5[d]	0.001
FFA	42.8	45.4	44.1	48.7	46.4	NS

[abcd] Values not sharing common superscripts per row differ significantly; NS, non-significant. Abbreviations: POL, polar lipid; DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty acids; HSG, high-sugar grass; C, control grass; RC, red clover.

2.5.3 Lipid Analysis Methods

Traditionally, thin-layer chromatography (TLC) has been used in the separation and analysis of lipids. The development of high-performance liquid chromatography (HPLC) methods for the analysis of lipids has seen considerable advances over recent years, however TLC is still preferred when dealing with complex lipids and wanting to achieve comprehensive separations (Christie, 2003).

A number of TLC methods have been used to fractionate grass lipids; varying from straightforward separations of neutral and polar lipids to more complex separation of individual galactolipids and phospholipids. Previous work carried out at Aberystwyth University has used the one-dimensional thin-layer chromatography (1D-TLC) method of Nichols (1963) to separate DAG, TAG, FFA and POL. Van Ranst *et al.* (2009), in contrast, used a two-stage solid-phase extraction (SPE) technique, based on Burdge *et al.* (2000) and Dreyfus *et al.* (1997), to separate ML, TAG + DAG and FFA.

In terms of detailed separation of lipids, many have used two-dimensional thin-layer chromatography (2D-TLC) to accomplish this. Yet, there is large variability between methods used, with no one method being obviously preferred over another. An overview of the types of methods found in the literature is given in Table 2.8.

Table 2.8 Overview of the methods used fractionate neutral lipids, galactolipids and phospholipids using thin-layer chromatography (TLC)

Reference	Extraction	Method	Plate details	Solvent A	Solvent B	Visualisation
Guschina *et al* (2014)	C:M (1:2)	2D-TLC	Silica gel G plates	C:M:W (65:25:4)	C:A:M:AA:W (50:20:10:10:5)	8-anilino-4-naphthosulphonic acid in methanol (0.05%) - UV
		1D-TLC		H:DEE:AA (80:20:1)	-	
Fraser *et al* (2004)	C:M:FA (10:10:1)	1D-TLC	Pre-coated silica gel plates	C:M:7MNH$_4$OH (65:30:4)	-	Iodine vapour
				C:M:25% Amm (60:30:7.5)	-	
Christie (2003)	-	-	20x20cm glass-backed	C:M:W (75:25:2.5)	C:M:AA:W (80:9:12:2)	-
Whitaker (1986)	Iso-P/ C:M (2:1)	SPE	Silicic Acid column 100-200 mesh bio-sil A	-	-	Distilled Water
		1D-TLC	20x20cm, Glass-backed, Silica gel 60, 0.25mm	A:AA:W (100:2:1)	-	
		1D-TLC		C:M:AA:W (85:15:10:3.5)	-	
Koiwai *et al* (1981)	C:M (1:2)	2D-TLC	-	C:M:W (65:25:4)	C:M:Iso-PA:Amm (65:35:0.5:5)	Rhodamine 6G solution - UV
Mackender and Leech (1974)	C:M (2:1)	2D-TLC	Silica gel H / HR	C:M:W (65:25:4)	A:AA:W (100:2:1)	2', 7'-dichlorofluorescein - UV
Rouser *et al* (1970)	-	2D-TLC	Silica Gel H - 1.5g Mg acetate	C:M:28% Amm$_{(Aq)}$ (65:25:5)	C:A:M:AA:W (3:4:1:1:0.5)	Charring
			silica Gel H - 0.5g Mg acetate	C:M:28% Amm$_{(Aq)}$ (65:35:5)	C:A:M:AA:W (5:2:1:1:0.5)	
Gardner (1968)	C:M (2:1)	2D-TLC	Silica Gel G, 250µm thick	C:M:W (65:25:4)	A:AA:W (100:2:1)	Iodine vapour
Lepage (1964)	C:M (2:1)	2D-TLC	20x20cm, glass-backed, Silica Gel G, 0.25mm	C:M:W (64:25:4)	DBK:AA:W (80:50:10)	Various

Abbreviations: A, acetone; AA, acetic acid; Amm, ammonia; Amm$_{(Aq)}$, aqueous ammonia; C, chloroform; DBK, di-isobutyl ketone; DEE, diethyl ether; FA, formic acid; H, hexane; Iso-P, isopropanol; Iso-PA, isopropyl-amine; M, methanol; W, water.

2.6 Fatty Acid Prediction Tools

Traditionally, methods such as gas chromatography (GC) and TLC are used to determine the FA and lipid content and composition of forages. However, these methods are typically time-consuming, destructive and expensive, may involve hazardous chemicals and require a skilled analytical technician (Foley *et al.*, 1998; Foster *et al.*, 2006). With the growing interest in FAs of forages, there is increased motivation to discover and develop non-destructive methods of determining FA content of forages which could yield instantaneous, real-time results.

2.6.1 Chlorophyll

Chlorophyll may provide an indirect method of estimating TFA content, due to the location of chlorophyll and a large proportion of FAs within the thylakoid membranes of chloroplasts. Consequently, positive correlations have been found between chlorophyll and TFA, and with C18:3n-3 (Hawke, 1973; Mayland *et al.*, 1976; Dierking *et al.*, 2010). The development of methods to estimate *in vivo* chlorophyll content enables real-time, non-destructive approximation of chlorophyll content. One such approach to *in vivo* chlorophyll estimation is the use of hand-held chlorophyll meters such as the *SPAD*-502 meter (Minolta, Japan). It operates by measuring the transmission of red and infra-red light through leaves using two light-emitting diodes (650 and 940nm), along with a photodiode detector (Markwell *et al.*, 1995). The leaf chlorophyll content is inversely proportional to the amount of light passing through the leaf and is calculated and displayed in arbitrary units. Chlorophyll meters have previously been used to monitor and improve N fertilisation management and efficiency in cereal crops such as maize, wheat and rice (Shapiro *et al.*, 2006; Varinderpal-Singh *et al.*, 2010; Errecart *et al.*, 2012). There have also been some

studies which have used this technology to predict nutritional characteristics such as N, CP, lignin, ADF and IVOMD in both temperate and tropical forages (Gáborčík, 2003; Errecart *et al.*, 2012; Hughes *et al.*, 2014). As such, *in vivo* chlorophyll methods may also be used to estimate FA content of forages, owing to the close association between these two characteristics.

2.6.2 Infrared Spectroscopy

Infrared (IR) spectroscopy offers a much more rapid, non-destructive and relatively low cost alternative to traditional laboratory analyses. Near-infrared reflectance (NIR) spectroscopy has been widely adopted and is now routinely used by the agricultural sector in the estimation of feed characteristics such as moisture content, CP, WSC, fibre (ADF, NDF and lignin) and IVOMD (Norris *et al.*, 1976; Abrams *et al.*, 1987; 1989; Baker and Barnes, 1990; Shenk and Westerhaus, 1994). It has also played a key role in the evaluation and improvement of the nutritional value of forage (Norris *et al.*, 1976; Abrams *et al.*, 1987; 1989; Baker and Barnes, 1990; Shenk and Westerhaus, 1994). A recent example of this is the work by Foskolos *et al.* (2015) who investigated the use of NIR spectroscopy to predict rumen degradability parameters of forages. Other IR spectroscopy methods, such as Fourier-transform mid-infrared (FTMIR) spectroscopy, have not been so widely investigated and used. However, previous studies have demonstrated that FTMIR spectroscopy is capable of predicting plant characteristics such as cell wall components, alkali index, C content, N content and CP content (Allison *et al.*, 2009a, 2009b; Belanche *et al.*, 2013). With the increasing interest in novel forage traits such as FA content and composition, it seems appropriate to investigate the capacity to which IR spectroscopy could assist in enhancing these traits.

2.7 Summary

To summarise, forage FAs are gaining interest due to the potential gains in (a) the nutritional value of ruminant products, (b) energy provision to ruminants which could lead to improved production efficiency and reduced GHG emission, and (c) provide an alternative non-oilseed biomass crop. The majority of studies have focussed on environmental and management effects, with a limited number of studies investigating species and variety differences and even fewer investigating genotypic differences. Studies investigating the lipid composition of forages are also very limited, with the bulk of this research dating back to the 1960s and 1970s. Increasing the knowledge and understanding of differences in FA content and composition at a genotypic level is valuable in terms of assessing the potential to selectively breed for this trait. Additionally, investigation into rapid, non-destructive methods to predict FA content could aid in accelerating the selection and breeding process.

The intentions of this thesis are to (a) assess genotypic and seasonal variation in FA and lipid content and composition; (b) investigate the effect variation in FA content has on other nutritional characteristics; (c) quantify the relationships between FA, lipids and other characteristics of perennial ryegrass; and (d) evaluate alternative methods to predict FA content of perennial ryegrass.

Chapter 3. Use of a Chlorophyll Meter to Predict Fatty Acid Content

3.1 Summary

Study investigating the use of *in vivo* measured chlorophyll content, estimated using a hand-held chlorophyll meter (*SPAD*-502), to predict FA content of perennial ryegrass. Two varieties selectively bred for divergent *SPAD* value were used in the study and maintained under glasshouse conditions. *In vivo* chlorophyll (*SPAD*) measurements and FA analysis was carried out on one harvest in December 2011. The two varieties differed significantly for *SPAD* value ($P<0.01$) and for C16:0 ($P<0.001$), C18:2n-6 ($P<0.01$), C18:3n-3 ($P<0.001$) and TFA content ($P<0.001$). Positive relationships were found between *SPAD* and C16:0 ($R^2 = 0.15$, $P<0.05$), C18:3n-3 ($R^2 = 0.19$, $P<0.01$) and TFA ($R^2 = 0.18$, $P<0.05$) yet these relationships were rather weak. Further investigation is needed to improve the relationship between *in vivo* chlorophyll content and FA content using a larger sample size, in addition to validation of the relationship between *SPAD* and *in vitro* chlorophyll content.

3.2 Introduction

Chlorophyll meters provide a straight-forward, instantaneous and non-destructive method to estimate chlorophyll content of plants. The most common application for chlorophyll meters is in improving the management and efficiency of N fertilisation of cereal crops such as maize, wheat and rice (Shapiro *et al.*, 2006; Varinderpal-Singh *et al.*, 2010; Errecart *et al.*, 2012) and to a lesser extent temperate forage grasses such as tall fescue and perennial ryegrass (Gáborčík, 2003; Errecart *et al.*, 2012). The *SPAD*-502 meter (Minolta, Japan; shown in Figure 3.1) measures the transmission of

red and infra-red light through leaves using two light-emitting diodes (650 and 940nm) along with a photodiode detector (Markwell *et al.*, 1995). The leaf chlorophyll content is inversely proportional to the amount of light passing through the leaf, as determined by the photodiode detector, and is displayed in arbitrary units.

Figure 3.1 *SPAD*-502 hand-held chlorophyll meter (Minolta, Japan)

As previously discussed in Chapter 2, the majority of plant FAs are contained within the thylakoid membranes of chloroplasts. Consequently, there is a positive correlation between FAs and chlorophyll (Hawke, 1973; Mayland *et al.*, 1976; Dierking *et al.*, 2010). The traditional methods used to determine lipid and/or FA content of forages, such as GC, are destructive and lengthy. With the growing interest in FA content and composition of forages, there is increased motivation to discover and develop non-destructive methods of determining FA content of forages. Chlorophyll meters may be a suitable candidate for this, owing to the location of both chlorophyll and the majority of FAs within chloroplasts. A preliminary study was set up with the aim of identifying whether two varieties of perennial ryegrass (*Lolium perenne*), with divergent *SPAD* values, actually differ in total and individual FA content.

3.3 Materials and Methods

3.3.1 Plants

Two varieties of perennial ryegrass derived from the B674G 13th generation breeding population were used in this study, which had been selectively bred for divergent *SPAD* value. Variety Ba14148 was the 'low' *SPAD* whereas Ba14149 was the 'high' *SPAD*. Twenty genotypes from each variety were selected at random with no replication. Individual plants were maintained in 4" pots in a Venlo for two months under ambient conditions prior to harvesting in December 2011.

3.3.2 Chlorophyll Meter (SPAD)

Chlorophyll content of live plants was predicted using a portable chlorophyll meter *SPAD*-502 (Konica Minolta, Japan) on the same day as harvesting. Twenty healthy leaves were randomly selected and measured; ensuring that selected leaves were a good representation of the whole plant. Measurements were taken at the mid-section of each leaf. The mean of these twenty measurements was recorded to give a single value per plant.

3.3.3 Sampling Procedure

Plants were harvested by hand to a height of ~5 cm between the hours of 13:00-15:00. All plant material was collected, bagged and temporarily stored on ice before being transported to the laboratory where sample fresh weight was recorded. Bagged samples were then freeze-dried (Edwards Super Modulyo, Severn Vacuum Services, Bristol, UK) under vacuum at -80^{o}C for approximately 96 hours. Samples were re-weighed immediately after freeze-drying to record freeze-dried weight. Dried samples were ground and homogenised using a Tecator Cyclotec 1093 (FOSS, Cheshire, UK) fitted with a 1mm screen then stored at -20^{o}C until further analysis. Fresh weight and

freeze-dried weight were used to calculate Freeze-dried matter as a percentage of fresh weight (FDM%).

3.3.4 Fatty Acid Determination

Fatty acid analysis was carried out using the one-step extraction and methylation procedure of Sukhija and Palmquist (1988). Approximately 0.45 g of ground freeze-dried sample was weighed into a culture tube and the weight recorded to four decimal places. Two ml of toluene, containing 0.5 mg/ml tricosanoic acid (C23:0) methyl ester as the internal standard (Sigma-Aldrich Co., USA), was accurately added to the tube followed by 3 ml of methanolic hydrochloride (HCl). Tubes were flushed with N, capped tightly and gently swirled to assure the sample was sufficiently wetted. Samples were then placed in a 70^{o}C water bath for 2 hours, with tubes swirled approximately every 15 min. Once cooled to room temperature, 5 ml of 6% potassium carbonate (w/v; carefully to avoid foaming) and 2 ml of toluene was added. Tubes were then capped, vortexed and centrifuged at 1500 rpm for 5 min using a Beckman J6-Mi centrifuge. The toluene layer containing the fatty acid methyl esters (FAMEs) was transferred to a second culture tube, in which approximately 1 g of anhydrous sodium sulphate and 1 g of activated charcoal had been added. The second culture tubes were capped and placed on an orbital shaker, at approximately 300 rpm for 20 min, to accelerate de-colouring, then centrifuged at 2000 rpm for 5 min. The toluene layer was transferred to pointed-end glass centrifuge tube using a glass Pasteur pipette. Pointed-end glass tubes were centrifuged at 1000 rpm for a further 5 min, to consolidate fine charcoal particles. Gas chromatography (GC) vials with 0.3 ml inserts were filled using a glass Pasteur pipette and capped.

Fatty acid methyl esters (FAMEs) were separated and quantified using a gas chromatography system fitted with a flame ionisation detector (GC-FID; CP-3800

with PAL Autosampler, Varian Inc., CA, USA) equipped with a CP-select 100 m x 0.25 mm chemically bonded for FAME column (Agilent technologies UK Ltd, Berkshire, England, UK). Samples were injected at a volume of 1 µl with a split ratio of 1:25 and He as the carrier gas at a flow rate of 1.5 ml/min (controlled by electronic flow control). The injection oven and FID temperatures were set at 250^{o}C and 255^{o}C, respectively. The column oven was programmed as follows: constant 70^{o}C for 0 min; 70^{o}C rising to 170^{o}C at 20^{o}C/min; constant 170^{o}C for 25 min; increase to 190^{o}C at 1^{o}C/min; hold for 0 min, increase to 230^{o}C at 2.7^{o}C/min; constant 230^{o}C for 3 min. Total run time was 68 min. Peaks were identified using a 37 FAME standard (S37, Supelco, Poole, Dorset, UK) and quantified using the internal standard. Varian Star v.6.41 software was used to capture and handle data.

3.3.5 Statistical Analysis

Data was analysed via GenStat (16th edition, VSN International Ltd, Hemel Hempstead, UK) using one-way analysis of variance (ANOVA) with variety as the fixed effect, Spearman's Rank correlation and linear regression.

3.4 Results

Observed SPAD values ranged from 35.1 to 55.7. Mean *SPAD* values for the Ba14148 'low' variety and the Ba14149 'high' variety were 45.5 and 49.5, respectively and differed significantly ($P<0.01$). Mean individual and TFA content of the two grass varieties are shown in Table 3.1. The Ba14149 'high' variety contained more TFA ($P<0.05$), C16:0 ($P<0.001$), C18:2n-6 ($P<0.01$) and C18:3n-3 ($P<0.001$). No significant difference was observed between the two varieties in terms of C18:0 and C18:1*cis*-9 content. Also, no differences were found between the two varieties in

terms of individual FA proportions, though proportion of C18:1*cis*-9 and C18:3n-3 were approaching significance (P<0.10).

Table 3.1 Comparison of individual and total fatty acid content (g kg^{-1} DM) of 'low' *SPAD* and 'high' *SPAD* perennial ryegrass varieties

Variety	**C16:0**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**
'Low'	4.81	0.4	0.62	4.07	18.84	31.32
'High'	5.94	0.46	0.7	4.98	24.79	39.97
s.e.d	0.326	0.031	0.047	0.305	1.636	2.376
P	***	NS	NS	**	***	***

NS, not significant; **P<0.01; ***P<0.001

Significant correlations were found between *SPAD* value and C16:0 (P<0.05), C18:3n-3 (P<0.01) and TFA (P<0.05) content. Figure 3.2, Figure 3.3 and Figure 3.4 illustrate respectively the linear ordinary least squares regression analyses between *SPAD* and C16:0, C18:3n-3 and TFA across both varieties. All relationships were found to be positive; with R^2 values of 0.15, 0.19 and 0.18 for *SPAD* vs. C16:0, C18:3n-3 and TFA, respectively.

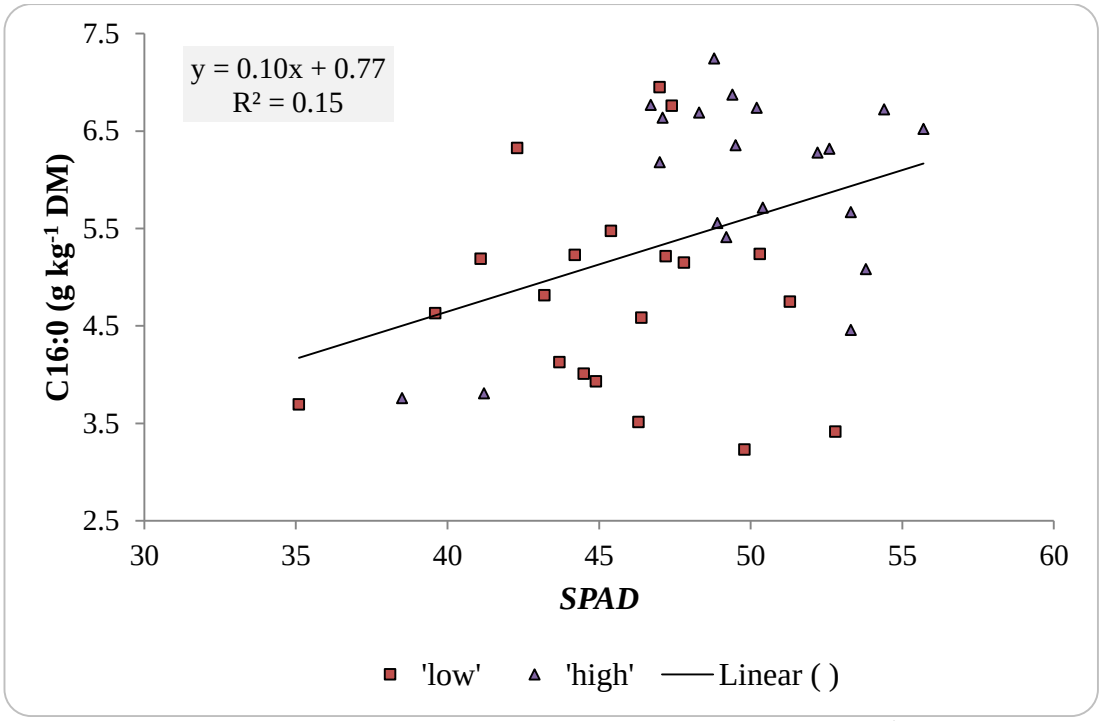

Figure 3.2 Relationship between *SPAD* (arbitrary units) and C16:0 (g kg^{-1} DM) across two populations of perennial ryegrass selected for divergent *SPAD*

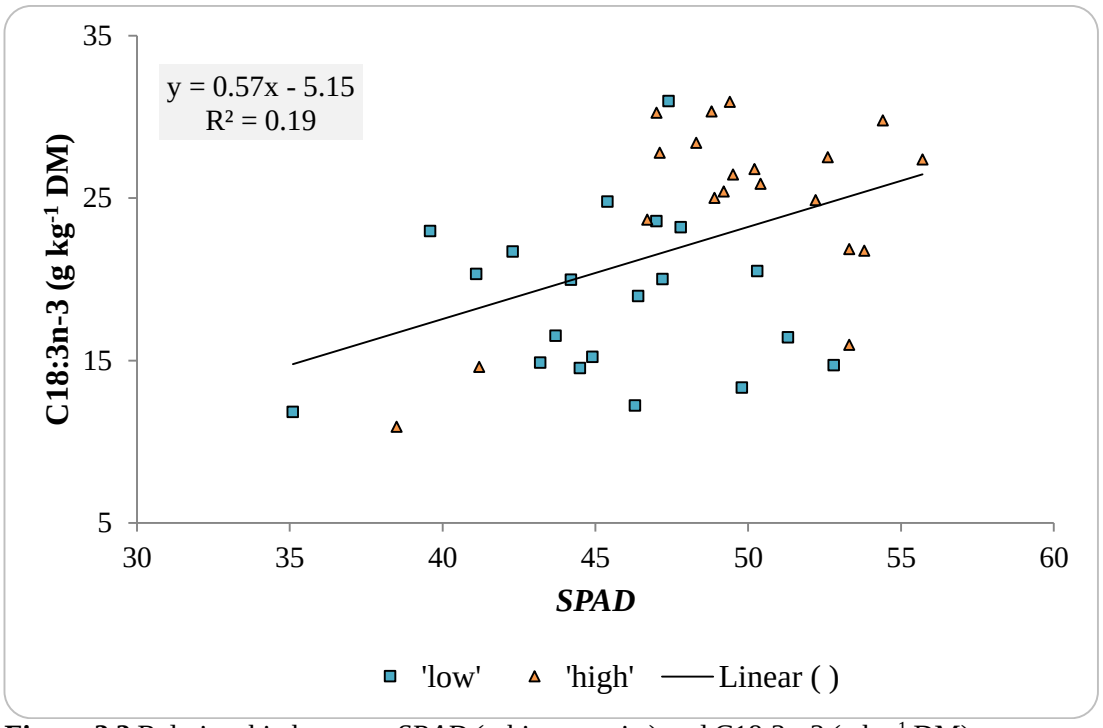

Figure 3.3 Relationship between *SPAD* (arbitrary units) and C18:3n-3 (g kg^{-1} DM) across two populations of perennial ryegrass selected for divergent *SPAD*

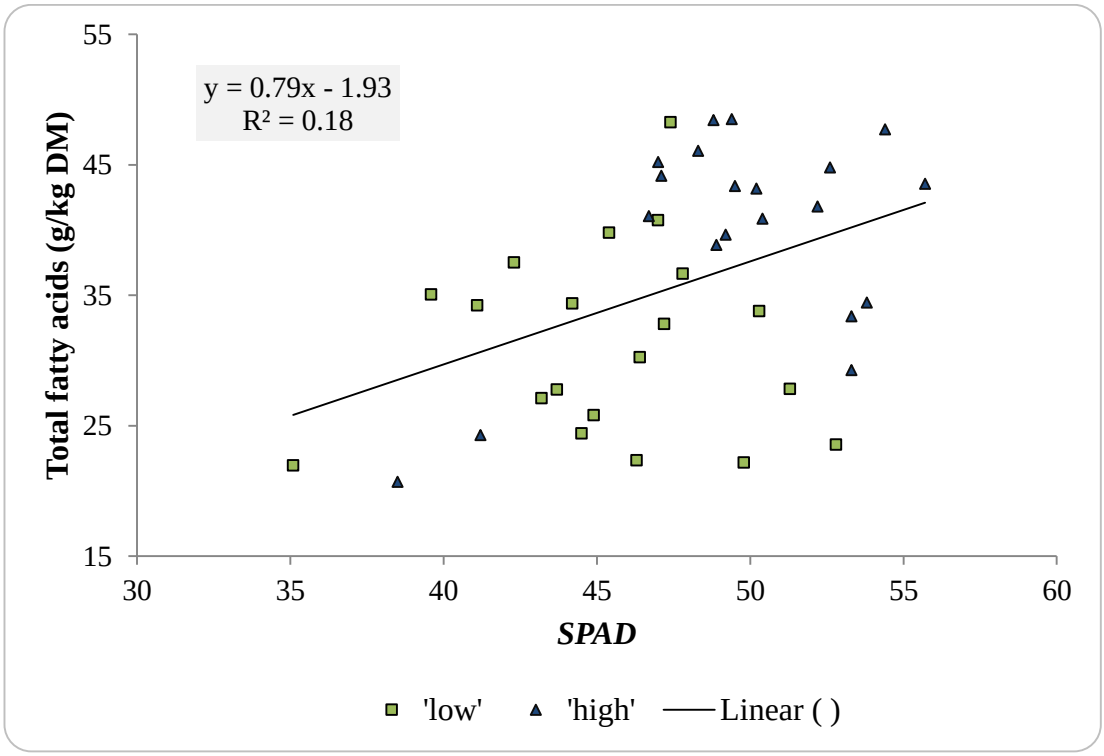

Figure 3.4 Relationship between *SPAD* (arbitrary units) and TFA (g kg^{-1} DM) across two populations of perennial ryegrass selected for divergent *SPAD*

3.5 Discussion

SPAD meter values typically range up to 50.0 (Ling *et al.*, 2011). The *SPAD* values found in the present study are towards the upper end of this range, as a result of the plants being relatively immature. The mean *SPAD* values found for both 'low' and 'high' *SPAD* varieties are comparable to values reported in other species. Dąbrowska (2013) included a greenness index of the leaf blade (*SPAD*) as one of the parameters when investigating the decorative value of *Festuca trachyphylla*, which was found to range from 33-45. Similar values have also been reported for *Festuca arundinacea* (44.3), *Lolium perenne* (43.5), *Lolium multiflorum* (40.4) and *Dactylis glomerata* (38.4) (Gáborčík, 2003; Olszewska *et al.*, 2008a).

The TFA content of both the 'low' *SPAD* and 'high' *SPAD* varieties was high but typical of young, leafy grass grown under glasshouse conditions. Likewise, Clapham *et al* (2005) found the TFA content of first harvest perennial ryegrass kept in similar conditions to be 5.2% DM. While Dierking *et al* (2010) reported the average TFA content of three harvests of perennial ryegrass as 4.4% DM. The TFA content of field-grown perennial ryegrass is usually lower when compared to more controlled environments such as a glasshouse. The studies by Dewhurst *et al* (2001) and Dewhurst *et al* (2002) reported the TFA content of perennial ryegrass harvested in November to be 2.4% DM and 3.5% DM, respectively. This lower TFA content during field conditions is probably due to added variability of environmental factors such as temperature, sunlight and rainfall, which are known to affect FAs (Hawke, 1973).

As expected, C18:3n-3 was the predominant FA in both varieties, accounting for over 60% of the TFA. Hawke (1973) reported that C18:3n-3 can account for 50-75% of the total lipid of forage. When considering a range of perennial ryegrass varieties, Dierking *et al* (2010) found C18:3n-3 proportion to vary between 65% and 71% TFA.

Regarding content of C16:0, Clapham *et al* (2005) and Dierking *et al* (2010) reported higher values compared to the present study. However, Clapham *et al* (2005) found comparable C18:2n-6 content, averaged across three different cuts, to the 'high' *SPAD* variety used in this study, yet Dierking *et al* (2010) reported a lower C18:2n-6 content of 3.89g kg^{-1} DM. These variances in FA composition may be due to genetic and environmental differences between studies.

The positive relationship found between *SPAD* and TFA, C18:3n-3 and C16:0 supports the notion of a positive relationship existing between chlorophyll and FA content. However the correlation coefficient (R^2) values are rather low, which may be due to the small sample size used in this study. In previous studies that investigated the relationship between *in vitro* chlorophyll content and TFA, correlation coefficients of up to 0.86 were reported for perennial ryegrass (Mayland *et al.*, 1976; Dierking *et al.*, 2010). Although the use of chlorophyll meters within agriculture and research is becoming more common, few studies have gone to the effort of quantifying and validating the relationship between *SPAD* output and *in vitro* chlorophyll content (Uddling *et al.*, 2007). The high R^2 values previously reported by Mayland *et al.* (1976) and Dierking *et al.* (2010) for *in vitro* chlorophyll and TFA content implies that the cause of the weak relationships between *in vivo* chlorophyll and FA content found in the present study may be due to poor prediction of chlorophyll content by the *SPAD*-502 meter.

3.6 Conclusions

These results show *in vivo* chlorophyll content, as predicted by a *SPAD*-502 meter, is positively associated with FA content in perennial ryegrass. This suggests that *SPAD* could potentially be used to approximate FA content. However, the relationships

between *SPAD* and FAs were rather weak, therefore *SPAD* may only be used as a vague estimate of FA content. Further analysis and quantification of the correlation between *SPAD* and *in vitro* chlorophyll content needs to be assessed, particularly with temperate forage species, where there is currently a lack of published research in comparison to cereal crops. Additionally, investigation of whether the relationship between *in vitro/in vivo* chlorophyll content and FA content changes at different plant growth stages or environmental conditions is also needed.

Chapter 4. Relationship between Fatty Acid Content and Nutritive Value

4.1 Summary

Twenty-four genotypes from two populations were used to assess the effect variation in FA content may have on other important nutritional characteristics of perennial ryegrass. Genotypes were selected based on historical *SPAD* value so that genotypes used in the study were representative of the FA variation within each population. Four replicates of each genotype were used, giving a total of ninety-six plants which were maintained under polytunnel conditions. Fatty acid, WSC and CP analysis was carried out on one harvest of these plants collected in July 2012. Significant variation was found between genotypes for FA content and proportions ($P<0.001$). Genotypes also differed for WSC content ($P<0.001$) but not CP content ($P>0.05$). Strong positive relationships were found between total and individual FA content with CP ($r = 0.31$ to 0.81; $P<0.01$). Conversely, negative relationships were found between total and individual FA content with WSC ($r = -0.25$ to -0.39, $P<0.05$) however these relationships were weaker. Additional investigation is needed to further confirm these relationships along with establishing relationships between FA content and other important characteristics such as fibre and digestibility.

4.2 Introduction

The range of grass breeding objectives have continuously expanded since the establishment of dedicated breeding locations, such as the Welsh Plant Breeding Station (WPBS), during the early 20th century. Initial objectives focused on agronomic characteristics such as yield, persistency and disease resistance (Wilkins and

Humphreys, 2003; Moorby *et al.*, 2008). Consideration was then given to the nutritional qualities of forage, starting with improvement of *in vitro* dry matter digestibility (IVDMD) and dry matter intake (DMI), followed more recently by WSC and CP. Breeding advances in ryegrass have been estimated as 4.0% per decade for WSC and 6.5% per generation for CP (Humphreys, 2005). Future plant breeding targets will also need to take into account increasing consumer demands for enhanced product quality and functionality (Kingston-Smith and Thomas, 2003).

The current interest in breeding for increased FA content in forage has arisen as a result of the substantial amount of evidence demonstrating the advantages of forage vs. grain feeding on the FA composition of ruminant products (see recent reviews by Kalač, 2011; Morgan *et al.*, 2012; Howes *et al.*, 2015). Increasing FAs could also improve the energy content of forage, which may have implications in terms of overall improved nitrogen utilisation and reduced methane emission (Winichayakul *et al.*, 2008; Ellis *et al.*, 2012). Furthermore, this may also provide an opportunity to develop non-seed biomass oil crops (Winichayakul *et al.*, 2013).

It is important to consider the relationships that exist between traits of interest, as some have been found to work antagonistically. For example, WSC content correlates negatively with CP/N content (Humphreys, 1989; Witkowska *et al.*, 2006). Only a limited number of studies have previously investigated the relationship between N or CP and FA content of forages, however those that have reported positive relationships between these two characteristics (Boufaïed *et al.*, 2003; Elgersma *et al.*, 2005). Fewer studies have investigated the relationship between FAs and WSC. This piece of work was carried out to further investigate these relationships and question the effect variation in FA content of perennial ryegrass would have on these nutritionally essential components.

4.3 Materials and Methods

4.3.1 Plants

Four control genotypes were selected from an Aurora x AberMagic F1 mapping population, which has been well characterised and previously been used to investigate FAs (Hegarty *et al*., 2013); along with twenty experimental genotypes selected from the B674G intermediate heading 13th generation breeding population. Genotypes were selected based on historic chlorophyll meter (*SPAD*) data, which has been shown to correlate positively with FA content (Chapter 3, Morgan *et al.*, 2013). This was in order to provide the best possible representation of the variation in FA content within each population (see Figure 4.1 for details). Mature single ryegrass tillers were transplanted into 6" pots in potting compost during April 2012. Four replicate clones of each genotype were used and were arranged in a randomised block design. Plants were maintained under poly-tunnel conditions, with actively reproductive heads cut back every two weeks in order to encourage tillering.

4.3.2 Sampling Procedure

Plants were harvested by hand in July 2012 between the hours of 12:00 and 18:00 to a cutting height of ~5 cm. All plant material was collected, bagged and temporarily stored on ice before transportation to the laboratory. Bagged samples were freeze-dried (Edwards Super Modulyo, Severn Vacuum Services, Bristol, UK) under vacuum at -80^{o}C for approximately 96 hours. Dried samples were ground and homogenised using a Tecator Cyclotec 1093 (FOSS, Cheshire, UK) fitted with a 1mm screen then stored at -20^{o}C until further analysis. Fresh and freeze-dried weights were recorded and FDM% calculated.

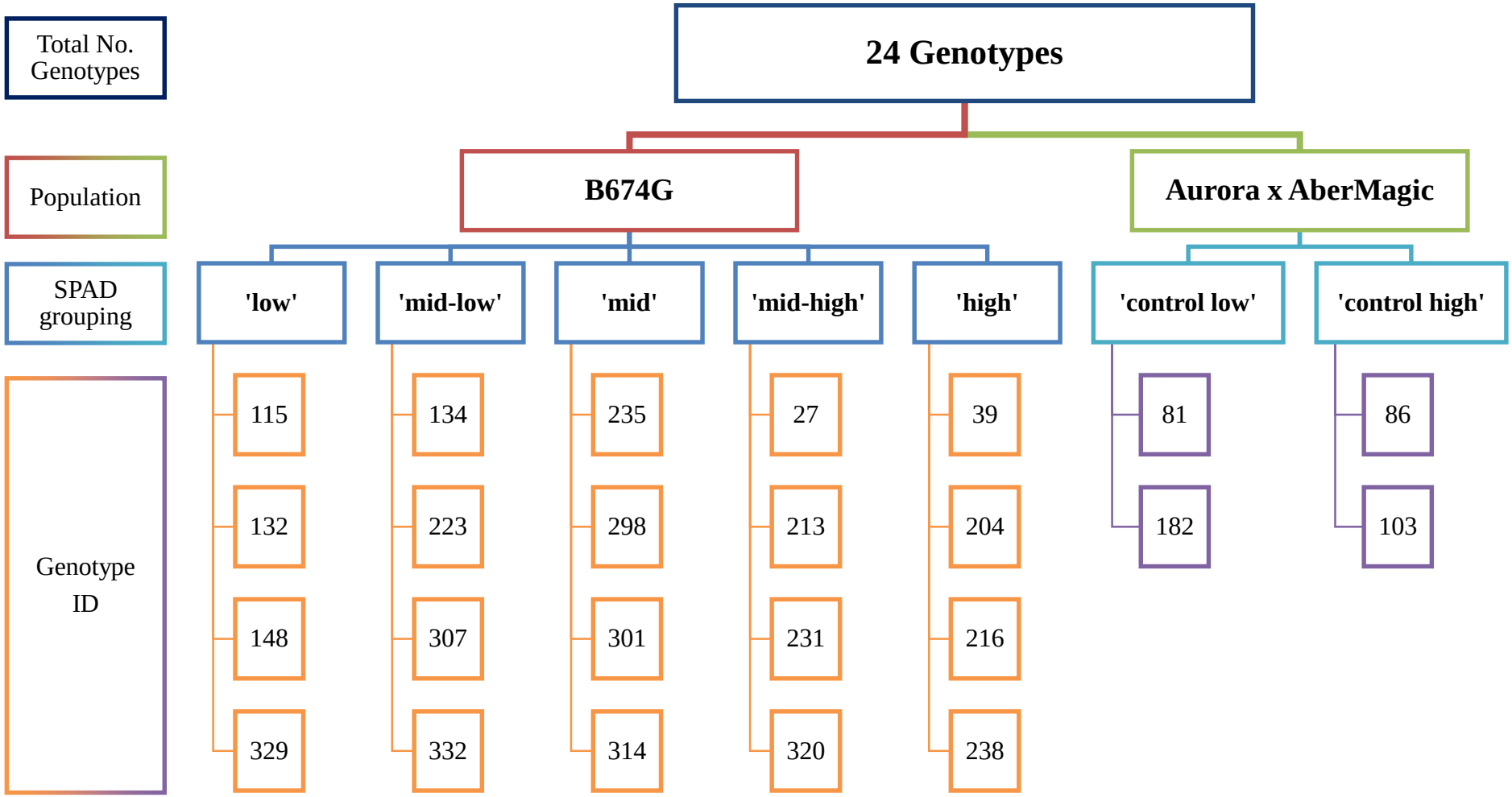

Figure 4.1 Schematic of the populations and genotypes selected for this study

4.3.3 CP and WSC Determination

Near-infrared reflectance (NIR) spectroscopy was used to estimate WSC and CP content. Approximately 2-5g of freeze-dried sample was packed into red cells and each scanned once at 2 nm intervals over the wavelength range from 400 to 2500 nm in reflectance mode, using a scanning monochromator (FOSS NIRSystems 6500, FOSS UK Ltd., Warrington, UK). Data were collected using WinISI II software (Version 1.02a, FOSS, Infrasoft International, Port Matilda, USA) and spectra were stored as log 1/R where R is the diffuse reflectance. Data over wavelength range 1100 to 2498 nm were used to develop calibrations for CP and WSC using WinISI 4 (Version 4.6.8, FOSS Analytical A/S).

4.3.4 Fatty Acid Determination

Fatty acid analysis was carried out as described in Chapter 3. Fatty acids were converted to methyl esters using the methylation procedure of Sukhija and Palmquist (1988) with C23:0 methyl ester used as the internal standard (Sigma-Aldrich Co., USA). Gas chromatography conditions for quantification of FAMEs were identical to those also described earlier in Chapter 3.

4.3.5 Statistical Analysis

Individual plant data were analysed via Genstat (16^{th} edition; VSN International Ltd, Hemel Hempstead, UK) using ANOVA to assess variation in CP, WSC and FA content between genotypes. Statistically different means were further analysed using Student-Newman-Keuls multiple comparisons test. Spearman's rank correlation was used to quantify the association between CP, WSC and FA content.

4.4 Results

4.4.1 Fatty Acid Content and Composition

Mean TFA content varied from 16.82 to 29.01 g kg^{-1} DM for genotypes from the B674G population, while mean TFA content for Aurora x AberMagic genotypes varied from 20.89 to 28.64 g kg^{-1} DM. Large and highly significant differences were found to exist between genotypes with regards to individual and total FA content ($P<0.001$; Table 4.1). The average TFA content across all genotypes from both populations was 23.23 g kg^{-1} DM (2.3% DM). The genotypes which resulted in the lowest and the highest TFA content were 314 and 298, respectively, both of which were from the B674G population.

Overall mean content of individual FAs was 3.93, 0.37, 0.38, 0.58, 3.64 and 12.80 g kg^{-1} DM for C16:0, C16:1*trans*-3, C18:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3, respectively. There was minimal difference between the two populations, apart from Aurora x AberMagic having a slightly higher average content across all genotypes for C16:0 and C18:3n-3 and lower C18:2n-6. Genotypes did not follow identical ranking orders for individual FAs relative to TFA content ranking. Genotype number 314 was lowest for C16:0, C16:1*trans*-3 and C18:3n-3 content. However, the genotypes with the highest content of these FAs were 86 for C16:0 and C18:3n-3, and 298 for C16:1*trans*-3. The lowest C18:0, C18:1*cis*-9 and C18:2n-6 contents were attributed to genotypes 332, 320 and 231, respectively, while the highest genotypes were 148, 213 and 298.

Large and significant differences were also found for FA proportions ($P<0.001$), as presented in Table 4.2. Genotype 314, which had the lowest TFA content, was found to have to lowest proportions of C18:3n-3 and C16:1*trans*-3 while having the highest

Table 4.1 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations

Population / Genotype		Fatty Acid Content (g kg^{-1} DM)						
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**
B674G	**27**	3.85 abc	0.30 abcd	0.41 cdef	0.53 ab	3.62 ab	12.00 abc	22.52 abcd
	39	3.53 abc	0.29 abcd	0.31 ab	0.56 abc	3.51 ab	9.95 ab	19.59 abc
	115	4.38 bc	0.38 abcde	0.43 cdef	0.65 bc	4.09 ab	13.78 abc	25.19 abcd
	132	4.14 abc	0.39 abcde	0.45 ef	0.63 abc	4.04 ab	13.97 abc	25.14 abcd
	134	4.17 bc	0.35 abcde	0.38 abcde	0.56 abc	3.80 ab	13.66 abc	24.38 abcd
	148	4.50 c	0.50 de	0.53 g	0.56 abc	3.67 ab	15.35 bc	26.61 bcd
	204	4.24 bc	0.39 abcde	0.34 abc	0.67 bc	4.11 ab	14.12 abc	25.37 abcd
	213	4.32 bc	0.39 abcde	0.42 cdef	0.71 c	4.21 ab	14.23 abc	25.87 abcd
	216	3.88 abc	0.42 bcde	0.39 bcdef	0.64 abc	3.54 ab	12.43 abc	23.05 abcd
	223	3.86 abc	0.34 abcde	0.36 abcde	0.50 ab	3.50 ab	11.86 abc	22.17 abcd
	231	3.39 ab	0.24 ab	0.30 a	0.54 abc	3.03 a	9.19 ab	18.14 ab
	235	3.76 abc	0.29 abcd	0.35 abc	0.53 ab	3.38 a	11.55 abc	21.51 abcd
	238	3.96 abc	0.32 abcde	0.41 cdef	0.57 abc	3.80 ab	11.83 abc	22.55 abcd
	298	4.29 bc	0.52 e	0.47 f	0.59 abc	4.56 b	16.85 c	29.01 d
	301	4.04 abc	0.34 abcde	0.40 bcdef	0.53 abc	3.71 ab	14.12 abc	24.82 abcd
	307	3.55 abc	0.34 abcde	0.34 abc	0.55 abc	3.53 ab	11.09 abc	20.91 abcd
	314	3.12 a	0.20 a	0.34 abc	0.66 bc	3.18 a	7.75 a	16.82 a
	320	3.33 ab	0.27 abc	0.35 abcd	0.47 a	3.07 a	9.98 ab	19.00 ab
	329	4.03 abc	0.45 bcde	0.39 bcdef	0.52 ab	3.84 ab	15.24 bc	26.23 bcd
	332	3.60 abc	0.31 abcde	0.29 a	0.61 abc	3.38 a	11.71 abc	21.35 abcd
Aurora x AberMagic	**81**	4.18 bc	0.45 bcde	0.35 abc	0.56 abc	3.60 ab	14.32 abc	24.94 abcd
	86	4.56 c	0.47 cde	0.44 def	0.62 abc	3.53 ab	17.36 c	28.64 cd
	103	4.01 abc	0.47 cde	0.42 cdef	0.59 abc	3.49 ab	13.68 abc	24.23 abcd
	182	3.70 abc	0.38 abcde	0.30 a	0.64 abc	3.20 a	11.25 abc	20.89 abcd
	s.e.d.	0.292	0.061	0.026	0.050	0.320	1.799	2.474
	P	***	***	***	***	***	***	***

***P<0.001; [abcgefg] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 4.2 Fatty acid proportions (% total FA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations

Population		**Fatty Acid Proportion (% total FA)**					
Genotype		**C16:0**	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	17.12 bcd	1.32 ab	1.82 abcde	2.34 ab	16.02 cdef	53.22 bc
	39	18.20 cd	1.44 abc	1.64 abcde	2.98 ab	18.03 gh	50.09 b
	115	17.52 bcd	1.51 abcdef	1.71 abcde	2.64 ab	16.39 cdefg	54.19 bc
	132	16.88 abcd	1.51 bcdef	1.86 bcde	2.71 ab	16.33 cdefg	54.14 bc
	134	17.32 bcd	1.41 ab	1.61 abcde	2.36 ab	15.69 cdef	55.31 bc
	148	16.94 abcd	1.87 g	2.01 de	2.10 ab	13.79 b	57.68 bc
	204	16.73 abcd	1.53 bcdef	1.36 a	2.67 ab	16.26 cdefg	55.51 bc
	213	16.86 abcd	1.48 abcdef	1.64 abcde	2.82 ab	16.43 cdefg	54.55 bc
	216	16.95 abcd	1.80 fg	1.72 abcde	2.81 ab	15.47 bcdef	53.38 bc
	223	17.82 bcd	1.43 abc	1.72 abcde	2.38 ab	16.18 cdefg	51.85 b
	231	18.68 d	1.32 ab	1.64 abcde	2.95 ab	16.72 defg	50.63 ab
	235	17.52 bcd	1.36 ab	1.62 abcde	2.44 ab	15.74 cdef	53.55 bc
	238	17.57 bcd	1.44 abcd	1.81 abcde	2.52 ab	16.83 efg	52.44 b
	298	14.84 a	1.78 cefg	1.62 abcde	2.07 ab	15.78 cdef	57.87 bc
	301	16.34 abcd	1.38 ab	1.61 abcde	2.15 ab	14.91 bcde	56.82 bc
	307	17.12 bcd	1.59 bcdefg	1.64 abcde	2.74 ab	17.09 fg	52.30 b
	314	18.58 d	1.14 a	2.04 e	4.04 c	19.05 h	45.66 a
	320	17.64 bcd	1.42 abc	1.88 cde	2.51 ab	16.35 cdefg	51.93 b
	329	15.45 ab	1.69 bcdefg	1.52 abc	2.00 a	14.65 bcd	57.80 bc
	332	16.89 abcd	1.45 abcde	1.38 a	2.85 ab	15.82 cdef	54.84 bc
Aurora x AberMagic	**81**	16.84 bcd	1.80 efg	1.40 ab	2.28 ab	14.56 bc	57.00 bc
	86	15.98 abc	1.62 bcdefg	1.55 abcd	2.18 ab	12.33 a	60.40 c
	103	16.56 abcd	1.91 g	1.76 abcde	2.45 ab	14.42 bc	56.34 bc
	182	17.72 bcd	1.82 fg	1.43 abc	3.12 b	15.39 bcdef	53.66 bc
	s.e.d.	0.650	0.106	0.129	0.284	0.585	2.101
	P	***	***	***	***	***	***

***P<0.001; [abcgefgh] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

proportions of C18:0, C18:1*cis*-9 and C18:2n-6. The genotypes with the highest proportions of C18:3n-3 and C16:1*trans*-3 were 86 and 103, respectively, while the lowest proportions of C18:0, C18:1*cis*-9 and C18:2n-6 were found in genotypes 204, 329 and 86. Proportion of C16:0 was highest in genotype 231 and lowest in 298.

4.4.2 CP and WSC Content

Results for CP and WSC content of genotypes is presented in Table 4.3. Mean CP content ranged from 99.1 to 158.4 g kg^{-1} DM, with genotypes from the Aurora x AberMagic population having slightly higher CP content than B674G genotypes. Overall mean CP content was 132.9 g kg^{-1} DM and no differences were found between genotypes for CP content ($P>0.05$). On the other hand, mean WSC content ranged from 152.4 to 257.4 g kg^{-1} DM and 114.4 to 192.6 g kg^{-1} DM for genotypes from the B674G and the Aurora x AberMagic populations, respectively. Overall mean WSC content was 188.1 g kg^{-1} DM, with significant differences found between genotypes ($P<0.001$). The genotype with the highest WSC content was 27, while the lowest was found to be 86.

4.4.3 Relationships between Fatty Acids, CP and WSC content

The Spearman's rank correlation results are presented in Table 4.4. All correlations between CP and FAs were positive. Very strong correlations of >0.8 were found between CP and C16:0, C16:1*trans*-3, C18:2n-6, C18:3n-3 and TFA content ($P<0.001$). The correlation between CP and C18:0 was slightly lower at 0.47 ($P<0.001$). The correlation between CP and C18:1*cis*-9 was the weakest correlation at 0.31 ($P<0.01$). Conversely, correlations between FA content and WSC content were all negative, however all were comparatively weaker than the correlations found

Table 4.3 Crude protein (CP) and water soluble carbohydrate (WSC) content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations

Population / Genotype		g kg^{-1} DM	
		CP	**WSC**
B674G	**27**	119.4	257.4 ^{c}
	39	119.2	166.4 ^{abc}
	115	143.6	173.0 ^{abc}
	132	134.5	178.6 ^{abc}
	134	149.4	177.1 ^{abc}
	148	149.4	170.9 ^{abc}
	204	153.8	174.5 ^{abc}
	213	144.7	229.3 ^{bc}
	216	134.8	186.9 ^{abc}
	223	129.7	152.4 ^{ab}
	231	119.8	179.2 ^{abc}
	235	110.9	222.2 ^{bc}
	238	126.9	187.0 ^{abc}
	298	153.6	180.8 ^{abc}
	301	146.4	187.5 ^{abc}
	307	121.6	224.3 ^{bc}
	314	100.0	247.7 ^{c}
	320	99.1	238.6 ^{bc}
	329	127.5	221.5 ^{bc}
	332	125.9	172.8 ^{abc}
Aurora x AberMagic	**81**	150.3	125.7 ^{a}
	86	158.4	114.4 ^{a}
	103	141.2	152.0 ^{ab}
	182	127.5	192.6 ^{abc}
	s.e.d.	18.51	25.32
	P	NS	***

NS, not significant; ***P<0.001; ^{abc} Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 4.4 Spearman's-rank correlation of crude protein (CP) and water-soluble carbohydrate (WSC) vs. individual and total fatty acid content (94 d.f.)

	CP	*WSC*
C16:0	**0.81**	**-0.39**
C16:1trans-3	**0.80**	**-0.37**
C18:0	**0.47**	**-0.25**
C18:1cis-9	**0.31**	**-0.26**
C18:2n-6	**0.77**	**-0.27**
C18:3n-3	**0.80**	**-0.35**
Total	**0.81**	**-0.35**

NB: **Bold** P<0.05; **Bold** P<0.01; **Bold** P<0.001

between CP and FAs. Stearic acid (C18:0) had the weakest correlation with WSC which was -0.25 ($P<0.05$). Marginally higher correlations were observed between WSC and C18:1*cis*-9, C18:2n-6 and C18:3n-3 ($P<0.01$). The strongest WSC correlations were with C16:0, C16:1*trans*-3 and TFA, which were -0.39, -0.37 and -0.35 ($P<0.001$), respectively. Additionally, a negative correlation of -0.62 was found between CP and WSC.

4.5 Discussion

The overall average TFA content observed in the present study is comparable to values published by Dewhurst *et al.* (2001, 2002), Elgersma *et al.* (2003a) and Van Ranst *et al.* (2009) for July harvests of perennial ryegrass. These studies reported values ranging from 2.2% DM to 2.6% DM, however lower values have also been previously reported (Dewhurst *et al.*, 2001). Species differences in terms of TFA content and FA profile have been previously reported (Dewhurst *et al.*, 2001; Boufaïed *et al.*, 2003; Clapham *et al.*, 2005). Focusing on July harvests in the study by Dewhurst *et al* (2001), *Lolium perenne* was found to have the highest TFA content (2.2% DM) while *Lolium multiflorum* had the lowest (1.5% DM).

Investigations into cultivar differences have also been carried out; for instance Elgersma *et al* (2003a, 2003b) reported small, non-significant differences between cultivars. They concluded that these differences were not due to heading date, yield, DM content or leaf blade proportion, and thus suggested the variation was indicative of the genetic basis of plant FA content. Palladino *et al* (2009), on the other hand, did report significant differences between cultivars. A more recent study by Hegarty *et al* (2013), investigating the genetic basis of FA content of perennial ryegrass in more detail, established that significant differences also exist between genotypes within the

same population. They concluded that although seasonal and environmental factors play a substantial role in FA content and composition, there is a significant genetic contribution to this, which is very encouraging in terms of the potential to selectively breed for this trait. The results of the present study adds further support to this notion of a strong genetic basis to FA content and composition, with genotype differences found for total and all 6 major individual FAs present in forage.

Linolenic acid (C18:3n-3) represents the highest proportion of TFA in forage, and typically accounts for around 50-75% of the TFA (Hawke, 1973; Dewhurst *et al.*, 2001; Elgersma *et al.*, 2003a; Van Ranst *et al.*, 2009). In this study, C18:3n-3 proportion was slightly lower, ranging from 45% to 60% TFA between genotypes with an overall average of 54%. Elgersma *et al* (2003a) reported considerably higher C18:3n-3 proportions of above 70% TFA and thus lower proportions of C16:0, C18:0, C18:1*cis*-9 and C18:2n-6 in comparison to the FA proportions found in the present study. The plants in their study were maintained under field conditions and were cut every 4-5 weeks whereas the plants used for this study were not cut for at least 3 months (apart from reproductive stems), which may explain the large difference in C18:3n-3 proportion between Elgersma *et al* (2003a) and the present study.

No difference was found between genotypes in terms of CP content yet significant variation was found for WSC content of genotypes. However, the WSC values found in the present study were lower than those reported by Clapham *et al* (2005) for an assortment of perennial ryegrass varieties, who found WSC content to be in the range of 204 to 305 g kg^{-1} DM for late July harvest.

Regarding the relationships between FA, WSC and CP content, a positive correlation was found between CP content and total and individual FA content. These

findings are in agreement with other studies which have investigated the relationship between N or CP and FA content. Elgersma *et al* (2005) found a very strong relationship between CP and C18:3n-3 (R^2 = 0.90, P<0.001) with similar results for CP and TFA relationship, while Boufaied *et al* (2003) reported a positive relationship between TFA and N content (R^2 = 0.79, P<0.001). Crude protein and WSC are known to have an inverse relationship (Humphreys, 1989), which was also observed in the present study. Accordingly WSC and FA content also showed inverse relationships. This may be due to the different locations of these constituents within plant cells. Results from the present study found slightly stronger relationships between FA and WSC content than Palladino *et al* (2009), who reported R^2 values of 0.04, 0.01 and 0.02 for TFA, C18:3n-3 and C18:2n-6 vs. WSC, respectively.

4.6 Conclusions

This study provides further evidence of the positive relationship between TFA and CP in perennial ryegrass. This study has also uncovered the negative relationship between TFA and WSC. Additional research is needed to further confirm these relationships, provide insight into the mechanisms underpinning them and establish how environment and season affect these relationships. Also, examining the relationships between FAs and other important characteristics will further establish the potential effects selecting for this trait may have on overall plant performance.

Chapter 5. Seasonal Effects on Chlorophyll, Fatty Acids and the Association between these Characteristics

5.1 Summary

An investigation into the genetic and seasonal variability of chlorophyll content, FA profile and the relationship between chlorophyll and FA content of perennial ryegrass. The same plants used in the previous study (Chapter 4) were transferred and maintained under field conditions. Five harvests collected between early June and late September 2013 were analysed for i*n vivo* chlorophyll (*SPAD*), *in vitro* chlorophyll and FA content and composition. In general, chlorophyll and FA content of leaf tissue increased during the growing season. Significant differences were found between genotypes for chlorophyll content, FA content and FA composition at every harvest; and these differences remained largely consist across the growing season. Positive relationships were found for both *in vivo* and *in vitro* chlorophyll with FA content. However, *in vivo* chlorophyll correlated poorly with both FA content and *in vitro* chlorophyll content. Using chlorophyll to predict FA content shows promise, however further work is needed to strengthen the relationships between *in vivo* estimated chlorophyll and *in vitro* chlorophyll content. Additionally, this work provides further evidence that differences in FA content and composition can exist at a genotypic level and are somewhat consistent across a growing season, supporting and strengthening the potential to selectively breed for this trait.

5.2 Introduction

Chlorophyll content is an important and frequently measured parameter of plants which is commonly used as an indicator of chloroplast development, photosynthetic capacity, leaf nitrogen content or general plant health (Ling *et al.*, 2011). Traditionally, chlorophyll content is measured in the laboratory using methods which are destructive, time-consuming and costly. Chlorophyll meters, such as the *SPAD*-502, provide an alternative approach to laboratory analysis which enables real-time, non-destructive approximation of chlorophyll content. As a result, numerous studies have investigated the potential for using non-destructive *in vivo* chlorophyll measures to predict other characteristics of plants. The most common example of this is in improving the management and efficiency of N fertilisation of cereal crops such as maize, wheat and rice (Shapiro *et al.*, 2006; Varinderpal-Singh *et al.*, 2010; Errecart *et al.*, 2012). The use of chlorophyll meters is less common with forage species. However, a limited number of studies have used chlorophyll meters to predict nutritional characteristics such as N, CP, lignin, ADF and IVOMD in both temperate and tropical forages (Gáborčík, 2003; Errecart *et al.*, 2012; Hughes *et al.*, 2014).

A close association exists between FAs and *in vitro* chlorophyll, as demonstrated by Hawke (1973), Mayland *et al* (1976) and Dierking *et al* (2010). Furthermore, the work presented in Chapter 3 also shows the potential in taking advantage of *in vivo* chlorophyll measures to estimate FA content. Building on this preliminary work, a larger field study was set up in order to gain more insight into genetic and seasonal variability in the chlorophyll content, FA profile and the relationship between chlorophyll and FA content of perennial ryegrass. The key aims of this study were to (i) assess the genetic variability in chlorophyll content and FA profile, (ii) assess the

seasonal effects on chlorophyll content and FA profile, and (iii) validate the relationships between *in vivo* chlorophyll, *in vitro* chlorophyll and FA content.

5.3 Materials and Methods

5.3.1 Plants

The plants used in this experiment have previously been described in Chapter 4. Briefly, they comprised of four Aurora x AberMagic F1 mapping population genotypes and twenty B674G intermediate heading 13th generation breeding population genotypes. The study was conducted at the Institute of Biological, Environmental and Rural Sciences (IBERS) site at Plas Gogerddan (52^{o}25'N, 4^{o}05'W). Plants were transferred from pots to the field in August 2012 and planted as spaced plants in a randomized block design.

Plants were subjected to a simulated grazing management regime which began in May 2013. The management schedule of the plants is presented in Table 5.1. Plant growth was poor during the beginning of 2013 due to low temperature and a lack of rainfall, especially in March and April (Figure 5.1); thus plants were cut but not sampled on the 7th May 2013 in order to encourage growth (see Table 5.1, 'cut –'). Plants were topped and fertilised with GrowHow MultiCut Sulphur 24-4-13 (7 SO_3) at the rate of 2.0 cwt/acre (55 kg/ha N) within one week after each harvest.

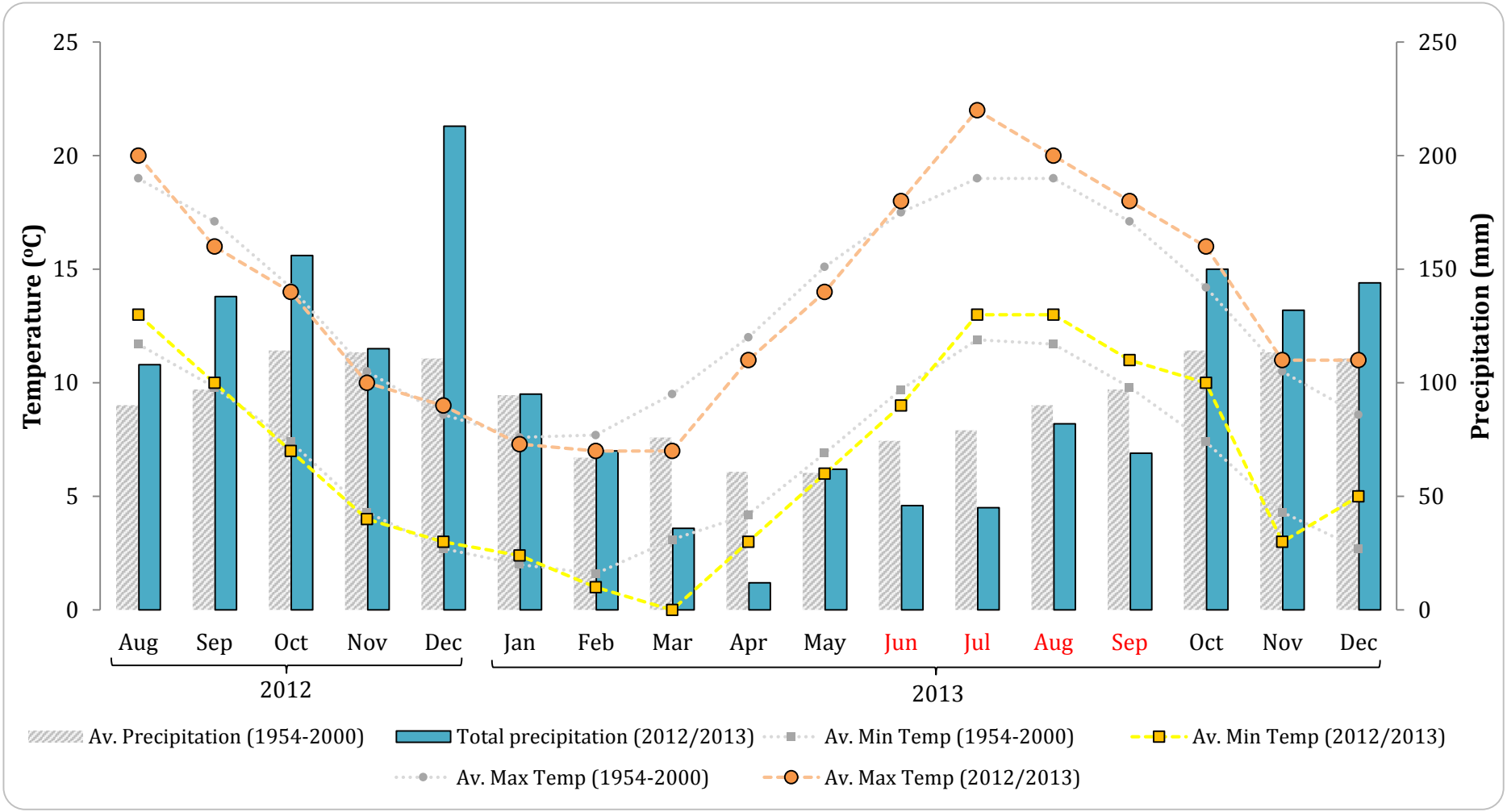

Figure 5.1 Averages per month for maximum temperature, minimum temperature and total precipitation for August 2012 to December 2013 along with historic monthly averages calculated for the period 1954 to 2000. Months when plants were sampled are highlighted in red.

Table 5.1 Harvesting and sampling regime of spaced plants investigate *SPAD*, chlorophyll and fatty acid variability and relationships across a growing season

Date	Cut No.	Description
07 May 2013	-	Topped (to encourage growth)
05 Jun 2013	1	Harvested and Sampled
01 Jul 2013	2	Harvested and Sampled
01 Aug 2013	3	Harvested and Sampled
28 Aug 2013	4	Harvested and Sampled
24 Sep 2013	5	Harvested and Sampled

5.3.2 Chlorophyll Meter (SPAD)

Chlorophyll content of live plants (*in vivo*) was estimated one day prior to harvesting using a portable chlorophyll meter *SPAD*-502 (Konica Minolta, Japan), details of which are given in Table 5.2. Twenty healthy leaves were randomly selected and measured; ensuring that selected leaves were a good representation of the whole plant. Measurements were taken at the mid-section of each leaf. The mean of these twenty measurements was then recorded to give a single value per plant.

Table 5.2 Dates and details of portable chlorophyll meter (*SPAD*) data collection

Date	Details
04 Jun 2013	Pre-Cut 1
30 Jun 2013	Pre-Cut 2
30 Jul 2013	Pre-Cut 3
27 Aug 2013	Pre-Cut 4
23 Sep 2013	Pre-Cut 5

5.3.3 Sampling Procedure

Plants were harvested by hand to a cutting height of ~5cm. Any senescent material and reproductive stems were removed from the sample before placing into a foil bag and snap freezing in liquid N then were temporarily stored on dry-ice in the field. Foil-wrapped samples were freeze-dried (Edwards Super Modulyo, Severn Vacuum Services, Bristol, UK) under vacuum at -80^{o}C for approximately 96 hours, ground and

homogenised using a Tecator Cyclotec 1093 (FOSS, Cheshire, UK) fitted with a 1mm screen then stored in the dark (to preserve chlorophyll) at -20ºC until further analysis. Fresh and freeze-dried weights were recorded and FDM% calculated.

5.3.4 Chlorophyll Determination

Direct chlorophyll content of freeze-dried plant material (*in vitro*) was determined using an acetone extraction procedure and analysed via spectrophotometry. Approximately 10 mg of ground freeze-dried sample was weighed into a 1.5 ml micro-centrifuge tube and the weight recorded to four decimal places. One ml of 80% acetone was added and tubes were left to extract overnight in darkness at -20ºC. The sediment was re-suspended and 0.5 ml transferred to a second micro-centrifuge tube, to which 0.5 ml 80% acetone was added, and then left to extract for a second time overnight in darkness at -20ºC. Tubes were centrifuged at 10,000 g and 4ºC for 10 min using a micro-centrifuge. Supernatant was transferred to a quartz cuvette and absorbance measured using an Ultrospec 4000 UV/Vis spectrophotometer (Pharmacia Biotech, New Jersey, USA) at wavelengths of 663nm (A_{663}) and 645nm (A_{645}). Total chlorophyll concentration (mg/l) was calculated as per Arnon (1949) as follows:

$$\mathbf{20.2\ (A_{645})\ +\ 8.02\ (A_{663})\ =\ mg/l}$$

Conversion of chlorophyll concentration (mg/l) to chlorophyll content (mg chl/g DM) was calculated as follows:

$$\frac{\mathbf{mg/l}}{\mathbf{1000}} = \mathbf{mg/ml}$$

$$\frac{\textbf{Chlorophyll concentration} \text{ (mg/ml)} \times \textbf{Total extract volume} \text{ (ml)}}{\textbf{Sample weight} \text{ (g)}} = \textbf{Chlorophyll content} \text{ (mg chl/g DM)}$$

5.3.5 Fatty Acid Determination

Analysis of FAs was carried out using the Sukhija and Palmquist (1988) methylation procedure followed by quantification via GC-FID, as described in detail in Chapter 3.

5.3.6 Statistical Analysis

Individual plant data were analysed via GenStat (16th edition, VSN International Ltd, Hemel Hempstead, UK). Within individual cuts, data were analysed using ANOVA as a randomised block design with genotype as treatment. Repeated measures ANOVA was used to analyse all genotypes and cuts at once. Statistically different means were further analysed using Student-Newman-Keuls multiple comparisons test. Associations between *in vivo* chlorophyll, *in vitro* chlorophyll and FAs were tested using Spearman's rank correlation.

5.4 Results

5.4.1 Chlorophyll Content

5.4.1.1 Chlorophyll Meter (SPAD)

Overall average *SPAD* values for Cuts 1 through to 5 were 33.7, 34.5, 41.6, 39.7 and 40.6, respectively, with the average of the four Aurora x AberMagic genotypes being slightly higher than the average of the B674G genotypes for all cuts. The overall lowest *SPAD* value was 26.7, recorded at Cut 1, while the highest *SPAD* value of 46.9 was recorded at Cut 5. Significant variation between genotypes was found at every cut ($P<0.01$), as shown in Table 5.3. However the multiple comparisons test failed to identify differences between individual genotype means at Cut 2, possibly due to groupings in the means (Thomas, 1973). Genotype 27 had the lowest *SPAD* value at

Cut 1 and Cut 2 while genotype 216 and 81 recorded the highest *SPAD* value for these cuts, respectively. Genotype 182 of the Aurora x AberMagic population resulted in the highest *SPAD* values for cuts 3, 4 and 5; however in Cut 4, genotype 216 had an equally high *SPAD* value to genotype 182. The lowest *SPAD* value for Cut 3 and Cut 4 was attributed to genotype 132. Yet, genotype 27 had an equally low *SPAD* value to genotype 132 at Cut 4 and also had the lowest *SPAD* value at Cut 5. When all cuts were examined together, the repeated measures ANOVA revealed highly significant cut and genotype effects ($P<0.001$), however no significant genotype x cut interaction was found (Table 5.5).

5.4.1.2 Chlorophyll Extraction

Highly significant genotype differences were found for chlorophyll content across all cuts ($P<0.001$), see Table 5.4 for details. Chlorophyll content generally increased from Cut 1 to Cut 5, with overall averages for each cut being 4.75, 5.97, 5.75, 6.38 and 7.98 for Cut 1 through to Cut 5, respectively. The highest chlorophyll content for all cuts was attributed to Aurora x AberMagic genotypes, with genotype 103 being highest at Cut 1 and genotype 86 being highest for the remaining cuts. On the other hand, B674G genotypes had the lowest chlorophyll content. Genotype 329 had the lowest chlorophyll content at Cut 1 while genotype 204 was the lowest ranked at Cut 2 and 3. For Cut 4 and Cut 5, the genotypes with the lowest chlorophyll content were 27 and 39, respectively. Highly significant effects of genotype, cut and genotype x cut ($P<0.001$) were also found when applying the repeated measures ANOVA statistical method (Table 5.5).

Table 5.3 Estimated chlorophyll content (*a+b*) (*SPAD* value) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations

Population / Genotype		**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**
B674G	**27**	26.7 a	30.0 a	41.6 ab	35.3 a	35.2 a
	39	32.6 abc	30.8 a	41.6 ab	36.9 abc	37.6 abcde
	115	29.4 ab	30.3 a	41.6 ab	36.4 ab	36.1 ab
	132	31.4 abc	31.0 a	34.5 a	35.3 a	35.7 a
	134	34.3 abc	33.7 a	42.5 b	40.9 abcde	43.6 efghi
	148	32.1 abc	33.3 a	41.4 ab	39.8 abcd	38.2 abcde
	204	33.3 abc	31.5 a	41.6 ab	37.9 abcd	36.9 abc
	213	31.0 abc	31.7 a	43.2 b	39.1 abcd	40.1 abcdefgh
	216	44.1 d	39.3 a	46.3 b	46.1 ef	46.0 hi
	223	33.5 abc	31.4 a	41.4 ab	41.8 bcdef	41.1 abcdefghi
	231	32.8 abc	34.0 a	44.5 b	38.3 abcd	38.8 abcdef
	235	33.7 abc	38.5 a	40.4 ab	41.3 abcdef	44.5 fghi
	238	33.2 abc	32.3 a	42.7 b	39.6 abcd	41.9 bcdefghi
	298	33.6 abc	37.7 a	40.3 ab	38.5 abcd	40.1 abcdefgh
	301	31.5 abc	37.1 a	43.5 b	40.6 abcd	43.5 efghi
	307	31.9 abc	34.6 a	39.8 b	38.4 abcd	39.5 abcdefg
	314	29.9 abc	30.2 a	40.8 ab	37.8 abcd	37.2 abcd
	320	32.4 abc	33.4 a	41.1 ab	37.3 abcd	38.1 abcde
	329	37.3 bc	33.8 a	39.2 b	39.9 abcd	40.4 abcdefgh
	332	39.2 cd	39.5 a	43.4 b	43.0 def	43.3 defghi
Aurora x AberMagic	**81**	36.6 bc	40.6 a	41.7 ab	42.5 cdef	45.1 ghi
	86	35.0 abc	36.8 a	40.4 ab	43.0 cdef	43.3 defghi
	103	36.7 bc	37.8 a	40.1 b	38.1 abcd	42.4 cdefghi
	182	36.6 bc	38.5 a	45.0 b	46.1 f	46.9 i
	s.e.d.	2.50	2.95	2.08	1.68	1.76
	P	***	***	**	***	***

**P<0.01; ***P<0.001; [abc] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.4. Total chlorophyll content (*a+b*) (mg chl/g DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations

Population / Genotype		**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**
B674G	**27**	3.68 ab	4.41 ab	4.92 abcd	4.31 a	6.77 a
	39	4.81 abcd	4.96 abc	4.53 abc	5.74 abcd	6.23 a
	115	3.99 abc	4.41 ab	4.47 abc	4.45 ab	6.93 a
	132	4.21 abcd	6.04 cd	4.77 abcd	4.86 abcd	7.27 ab
	134	4.33 abcd	5.58 bcd	5.26 abcde	6.19 bcde	8.12 abcd
	148	5.00 bcde	5.76 bcd	6.26 efghij	6.53 cde	7.47 ab
	204	4.36 abcd	3.83 a	4.07 a	4.92 abcd	6.30 a
	213	5.10 bcde	5.24 bcd	4.23 ab	5.02 abcd	6.50 a
	216	4.76 abcd	6.49 cdef	4.86 abcd	6.30 bcde	8.12 abcde
	223	4.26 abcd	5.55 bcd	5.46 bcdefg	6.28 bcde	7.77 ab
	231	5.20 cdef	5.62 bcd	5.39 bcdef	5.46 abcd	7.59 ab
	235	4.60 abcd	6.58 cdefg	5.89 defghij	6.40 cde	8.05 abc
	238	4.21 abcd	5.94 bcd	5.86 defghij	5.67 abcd	7.73 ab
	298	5.55 def	7.75 efgh	8.27 k	8.13 gh	9.48 bcdef
	301	4.04 abc	6.32 cde	6.65 fghij	6.70 def	8.18 abcde
	307	4.29 abcd	5.41 bcd	4.68 abcd	5.50 abcd	6.43 a
	314	4.15 abcd	5.61 bcd	4.41 abc	4.71 abc	6.36 a
	320	4.39 abcd	5.76 bcd	5.49 bcdefgh	5.43 abcd	6.50 a
	329	3.55 a	5.56 bcd	5.62 cdefghi	5.40 abcd	6.97 a
	332	5.29 cdef	6.55 cdef	6.94 j	8.10 gh	9.39 bcdef
Aurora x AberMagic	**81**	6.15 efg	7.72 fh	7.89 k	9.41 h	11.48 g
	86	6.29 fg	9.03 i	8.64 k	10.82 i	11.58 g
	103	6.92 g	6.81 defgh	6.65 gij	7.80 eg	9.97 cdefg
	182	4.80 abcd	6.43 cdef	6.70 ghij	8.99 gh	10.21 dfg
	s.e.d	0.399	0.458	0.394	0.537	0.690
	P	***	***	***	***	***

**P<0.01; ***P<0.001; [abcdefghijk] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.5 Repeated measures ANOVA output showing genotype, cut and genotype x cut interaction effects

	Genotype		Cut		Genotype x Cut	
	s.e.d.	**P**	**s.e.d.**	**P**	**s.e.d.**	**P**
SPAD	1.43	***	0.44	***	2.40	NS
Chlorophyll	0.316	***	0.092	***	0.513	***

NS, Not significant; ***P<0.001

5.4.1.3 Chlorophyll Meter (SPAD) vs. Chlorophyll Extraction

As anticipated, positive correlations were found between *SPAD* and chlorophyll content, the results of which are summarised in Table 5.6. Moderate correlations of 0.49, 0.54 and 0.66 (P<0.001) were found for Cut 2, Cut 4 and Cut 5, respectively, with Cut 5 resulting in the strongest correlation overall. However, a weak correlation was observed for Cut 1 (0.24, P<0.05) and practically no relationship was detected at Cut 3 (0.08, P>0.05). The across cut correlation between these two methods of measuring chlorophyll was 0.52 (P<0.001).

Table 5.6 Spearman's Rank correlations between chlorophyll meter (*SPAD*) and chlorophyll content (mg Chl/g DM)

Chlorophyll meter (SPAD)	*Chlorophyll extraction*
Cut 1	**0.24 (93)***
Cut 2	**0.49 (93)**
Cut 3	0.08 (72)
Cut 4	**0.54 (91)**
Cut 5	**0.66 (92)**
All	**0.52 (449)**

*Degrees of freedom; **Bold** P<0.05; **Bold** P<0.001

5.4.2 Fatty Acid Content and Composition

5.4.2.1 Genotype Performance within Individual Cuts

♦ *Cut 1 (Early June)*

Fatty acid content and composition of genotypes at Cut 1 harvest are presented in Table 5.7 and Table 5.8, respectively. Highly significant genotype differences were observed for TFA and all major individual FAs content as well as individual FA proportions ($P<0.001$). Overall average TFA content was 2.9% DM (29.0 g kg^{-1} DM). Genotypes from the Aurora x AberMagic population had the highest content of C16:0, C16:1*trans*-3, C18:1*cis*-9, C18:3n-3 and TFA while having the lowest content of C18:0 and C18:2n-6. The opposite was true for genotypes of the B674G population which had the lowest C16:0, C16:1*trans*-3, C18:1*cis*-9, C18:3n-3 and TFA content and the highest C18:0 and C18:2n-6 content.

Palmitic acid (C16:0) ranged from 3.43 to 4.42 g kg^{-1} DM, with genotype 329 the lowest while genotype 81 was the highest. Genotype 329 also had the lowest content of C16:1*trans*-3 (0.35 g kg^{-1} DM) whereas genotype 103 had the highest content at 0.82 g kg^{-1} DM. The lowest C18:0 content was with genotype 182 at 0.24 g kg^{-1} DM while genotype 148 had the highest content of C18:0 at 0.43 g kg^{-1} DM. Oleic acid (C18:1*cis*-9) ranged from 0.44 to 0.66 g kg^{-1} DM with genotypes 134 and 103 having the lowest and highest content of this FA, respectively. Genotype 182 also had the lowest C18:2n-6 content (2.62 g kg^{-1} DM) as well as the lowest C18:0 content, the highest C18:2n-6 content was genotype 204 (3.73 g kg^{-1} DM). The lowest C18:3n-3 and TFA content was seen in genotype 329, which also had the lowest C16:0 and C16:1*trans*-3 content. The highest content of C18:3n-3 and TFA was noted for genotype 81 and 103, respectively.

Table 5.7 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (first cut)

Population / Genotype		Fatty Acid (g kg^{-1} DM)						
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**
B674G	**27**	3.63 ^{abc}	0.40 ^{abc}	0.36 ^{cdef}	0.49 ^{abc}	3.24 ^{abcde}	15.90 ^{a}	26.20 ^{ab}
	39	4.03 ^{abcd}	0.56 ^{def}	0.38 ^{cdefg}	0.55 ^{abcd}	3.57 ^{cde}	18.80 ^{a}	29.92 ^{ab}
	115	3.91 ^{abcd}	0.39 ^{ab}	0.35 ^{cd}	0.60 ^{bcd}	3.32 ^{bcde}	16.23 ^{a}	26.91 ^{ab}
	132	3.80 ^{abcd}	0.37 ^{ab}	0.35 ^{cd}	0.64 ^{cd}	3.27 ^{abcde}	16.07 ^{a}	26.23 ^{ab}
	134	3.82 ^{abcd}	0.42 ^{abc}	0.35 ^{cd}	0.44 ^{a}	2.99 ^{abcd}	17.19 ^{a}	27.12 ^{ab}
	148	3.98 ^{abcd}	0.62 ^{fg}	0.43 ^{eg}	0.48 ^{ab}	3.26 ^{abcde}	19.25 ^{a}	30.12 ^{ab}
	204	3.95 ^{abcd}	0.46 ^{abcd}	0.34 ^{cd}	0.52 ^{abcd}	3.73 ^{e}	17.04 ^{a}	28.12 ^{ab}
	213	4.12 ^{abcd}	0.54 ^{cdef}	0.39 ^{defg}	0.53 ^{abcd}	3.65 ^{de}	19.37 ^{a}	31.00 ^{bc}
	216	3.85 ^{abcd}	0.51 ^{bcdef}	0.26 ^{ab}	0.65 ^{d}	3.07 ^{abcde}	17.65 ^{a}	28.14 ^{ab}
	223	3.85 ^{abcd}	0.52 ^{bcdef}	0.35 ^{cd}	0.48 ^{ab}	3.22 ^{abcde}	17.97 ^{a}	28.44 ^{ab}
	231	4.05 ^{abcd}	0.49 ^{abcde}	0.34 ^{cd}	0.52 ^{abcd}	3.04 ^{abcde}	18.85 ^{a}	29.25 ^{ab}
	235	3.53 ^{ab}	0.36 ^{a}	0.30 ^{bc}	0.46 ^{ab}	2.82 ^{ab}	15.86 ^{a}	25.38 ^{ab}
	238	3.98 ^{abcd}	0.46 ^{abcd}	0.34 ^{cd}	0.49 ^{abc}	3.36 ^{bcde}	18.19 ^{a}	28.95 ^{ab}
	298	4.21 ^{bcd}	0.61 ^{efg}	0.38 ^{cdefg}	0.58 ^{abcd}	3.60 ^{de}	22.69 ^{b}	34.34 ^{cd}
	301	3.74 ^{abcd}	0.45 ^{abcd}	0.31 ^{abc}	0.55 ^{abcd}	2.88 ^{abc}	17.51 ^{a}	27.36 ^{ab}
	307	3.84 ^{abcd}	0.46 ^{abcd}	0.34 ^{cd}	0.52 ^{abcd}	3.26 ^{abcde}	17.78 ^{a}	28.11 ^{ab}
	314	3.60 ^{abc}	0.39 ^{ab}	0.36 ^{cd}	0.64 ^{d}	3.40 ^{bcde}	15.79 ^{a}	26.25 ^{ab}
	320	3.88 ^{abcd}	0.47 ^{abcd}	0.36 ^{cdef}	0.52 ^{abcd}	3.15 ^{abcde}	17.27 ^{a}	27.59 ^{ab}
	329	3.43 ^{a}	0.35 ^{a}	0.26 ^{ab}	0.53 ^{abcd}	2.98 ^{abcd}	15.03 ^{a}	24.52 ^{a}
	332	3.54 ^{ab}	0.45 ^{abcd}	0.27 ^{ab}	0.46 ^{ab}	2.73 ^{ab}	18.16 ^{a}	27.54 ^{ab}
Aurora x AberMaaic	**81**	4.42 ^{d}	0.79 ^{h}	0.35 ^{cd}	0.55 ^{abcd}	3.02 ^{abcd}	24.26 ^{b}	35.66 ^{d}
	86	4.32 ^{cd}	0.70 ^{g}	0.34 ^{cd}	0.61 ^{bcd}	3.19 ^{abcde}	23.54 ^{b}	34.75 ^{cd}
	103	4.27 ^{bcd}	0.82 ^{h}	0.36 ^{cde}	0.66 ^{d}	3.52 ^{cde}	23.97 ^{b}	35.81 ^{d}
	182	3.53 ^{ab}	0.62 ^{efg}	0.24 ^{a}	0.54 ^{abcd}	2.62 ^{a}	18.56 ^{a}	28.26 ^{ab}
	s.e.d.	0.203	0.042	0.022	0.042	0.192	1.216	1.672
	P	***	***	***	***	***	***	***

***P<0.001; ^{abcgefgh} Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.8 Fatty acid composition (% TFA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (first cut)

Population / Genotype		Fatty Acids (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	13.87 def	1.55 abcd	1.40 jk	1.84 abc	12.33 ghij	60.63 abc
	39	13.49 cd	1.87 ef	1.28 efghijk	1.83 abc	11.94 efghi	62.66 abcde
	115	14.55 f	1.44 a	1.29 fghijk	2.25 gh	12.34 fghij	60.31 ab
	132	14.50 ef	1.42 a	1.34 hijk	2.44 h	12.49 hij	61.18 abcd
	134	14.10 def	1.54 abc	1.31 ghijk	1.63 ab	11.03 cdefg	63.37 cdefgh
	148	13.21 cd	2.07 gh	1.42 k	1.59 a	10.83 cde	63.88 defghi
	204	14.05 def	1.65 abcde	1.19 defghij	1.85 abc	13.24 j	60.61 abc
	213	13.32 cd	1.74 bcde	1.26 efghijk	1.70 ab	11.82 defghi	62.37 abcde
	216	13.69 def	1.82 def	0.92 ab	2.32 gh	10.93 cdef	62.66 abcde
	223	13.56 de	1.83 def	1.25 efghijk	1.68 ab	11.32 defgh	63.21 bcdef
	231	13.87 def	1.68 abcde	1.15 cdefgh	1.79 ab	10.42 cd	64.34 efghi
	235	13.88 def	1.43 a	1.20 defghij	1.82 abc	11.08 cdefg	62.51 abcde
	238	13.74 def	1.60 abcde	1.18 defghi	1.70 ab	11.60 defgh	62.84 abcde
	298	12.30 ab	1.78 cde	1.10 bcdef	1.69 ab	10.51 cd	65.97 fhij
	301	13.70 def	1.65 abcde	1.12 cdefg	2.03 bcdefg	10.55 cd	63.89 defghi
	307	13.68 def	1.64 abcde	1.22 efghij	1.86 abcd	11.60 defgh	63.26 bcdefg
	314	13.71 def	1.49 ab	1.36 ijk	2.45 h	12.94 ij	60.15 a
	320	14.09 def	1.69 abcde	1.32 ghijk	1.90 abcdef	11.45 defgh	62.52 abcde
	329	14.00 def	1.45 a	1.08 bcde	2.18 cegh	12.22 fghij	61.22 abcd
	332	12.80 bc	1.63 abcde	0.97 abc	1.65 ab	9.88 bc	65.96 ij
Aurora x AberMagic	**81**	12.38 ab	2.22 gh	0.99 abc	1.53 a	8.48 a	67.97 j
	86	12.45 ab	2.02 fg	0.97 abc	1.76 ab	9.18 ab	67.66 j
	103	11.91 a	2.28 h	1.01 abcd	1.82 abc	9.78 bc	66.92 j
	182	12.48 ab	2.21 gh	0.86 a	1.89 abcde	9.27 ab	65.72 fghij
	s.e.d.	0.269	0.082	0.061	0.113	0.416	0.866
	P	***	***	***	***	***	***

***P<0.001; [abcgefghijk] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

As previously stated, differences between genotypes were also found for individual FA proportions (P<0.001). Proportion of C16:0 ranged from 11.91 to 14.55% TFA, with genotype 103 and 115 having the lowest and highest proportions of this FA, respectively. On the contrary, genotype 103 had the highest C16:1*trans*-3 proportion of 2.28% TFA while genotype 132 had the lowest C16:1*trans*-3 proportion of 1.42% TFA. Stearic acid (C18:0) proportion ranged from 0.86 to 1.42% TFA with genotype 182 having the lowest proportion and genotype 148 having the highest proportion. The proportions of C18:1*cis*-9, C18:2n-6 and C18:3n-3 ranged from 1.53 to 2.45% TFA, 8.48 to 13.24% TFA and 60.15 to 67.97% TFA, respectively. Genotype 81 had the lowest proportions of C18:1*cis*-9 and C18:2n-6 and the highest proportion of C18:3n-3. The highest C18:1*cis*-9 proportion was attributed to genotype 314 while the highest C18:2n-6 proportion was by genotype 204. Genotype 314 also had the lowest C18:3n-3 proportion.

♦ *Cut 2 (Early July)*

Highly significant genotype differences were present at Cut 2 for both FA content and composition (P<0.001), although ranking of genotypes was slightly different to Cut 1. Fatty acid content and composition data for this cut is presented in Table 5.9 and Table 5.10. Overall average TFA content had increased from 2.9 to 3.2% DM (31.72 g kg^{-1} DM) from Cut 1 to Cut 2. Genotypes from the Aurora x AberMagic population were again highest for C16:0, C16:1*trans*-3, C18:3n-3 and TFA and lowest for C18:0 and C18:2n-6.

Palmitic acid (C16:0) content ranged from 3.91 to 5.01 g kg^{-1} DM, with genotypes 27 and 86 having the lowest and highest content of this FA, respectively. Genotype 81 has the highest C16:1*trans*-3 content at 0.73 g kg^{-1} DM while genotype 204 had the

Table 5.9 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (second cut)

Population / Genotype		Fatty Acid (g kg^{-1} DM)													
		C16:0		**C16:1t3**		**C18:0**		**C18:1c9**		**C18:2n-6**		**C18:3n-3**		**Total**	
B674G	**27**	3.91	a	0.42	ab	0.36	bcde	0.47	abc	3.62	abc	16.60	ab	27.26	a
	39	4.10	abcd	0.52	bcde	0.38	defgh	0.51	abcd	3.67	abc	17.68	abc	28.60	abc
	115	4.18	abcd	0.44	abc	0.44	hij	0.60	bcd	3.74	abc	16.65	ab	27.78	ab
	132	4.32	abcd	0.53	bcde	0.46	ij	0.60	bcd	3.71	abc	20.46	cde	31.89	abcde
	134	4.39	abcd	0.50	abcd	0.40	defgh	0.54	abcd	3.78	abc	19.34	bcde	30.53	abcd
	148	4.52	abcde	0.66	fg	0.48	j	0.54	abcd	3.76	abc	21.19	cde	32.74	bcde
	204	4.27	abcd	0.40	a	0.34	abcd	0.76	e	4.46	d	15.70	a	27.90	ab
	213	4.33	abcd	0.54	cde	0.39	defgh	0.51	abcd	3.84	bc	19.34	abcde	30.80	abcd
	216	4.33	abcd	0.63	efg	0.31	abc	0.66	def	3.38	abc	21.24	cde	32.54	bcde
	223	4.14	abcd	0.53	bcde	0.39	defgh	0.41	a	3.54	abc	18.67	abcd	29.42	abc
	231	4.14	abcd	0.50	abcd	0.37	bdefg	0.50	abcd	3.19	a	18.63	abcd	29.06	abc
	235	4.64	bcde	0.57	def	0.42	efghi	0.56	abcd	3.54	abc	21.89	de	33.46	cde
	238	4.69	cde	0.57	def	0.37	bcdefg	0.53	abcd	3.78	abc	21.78	de	33.63	cde
	298	4.74	de	0.59	def	0.44	ghij	0.65	de	4.29	d	25.21	fg	37.84	fg
	301	4.35	abcd	0.54	cde	0.39	defgh	0.51	abcd	3.42	abc	21.26	cde	32.31	abcde
	307	4.27	abcd	0.51	abcd	0.38	defgh	0.54	abcd	3.61	abc	18.82	abcd	29.84	abc
	314	4.04	abc	0.44	abc	0.44	fghij	0.62	cd	3.71	abc	19.08	abcde	30.33	abcd
	320	4.17	abcd	0.49	abcd	0.41	efghi	0.45	abc	3.21	ab	20.04	bcde	30.45	abcd
	329	4.01	ab	0.49	abcd	0.37	bcdef	0.43	ab	3.23	ab	19.45	bcde	29.90	abc
	332	4.44	abcd	0.52	abcde	0.31	ab	0.60	bcd	3.51	abc	21.46	cde	32.60	bcde
Aurora x AberMagic	**81**	4.76	de	0.73	g	0.38	defgh	0.53	abcd	3.18	a	25.01	fg	36.39	efg
	86	5.01	e	0.71	g	0.40	defghi	0.55	abcd	3.40	abc	27.17	g	39.23	g
	103	4.60	bcde	0.69	g	0.40	defgh	0.61	cd	3.90	c	22.92	ef	35.04	def
	182	4.13	abcd	0.72	g	0.30	a	0.56	abcd	3.17	a	21.37	cde	31.81	abcde
	s.e.d.	0.181		0.036		0.020		0.048		0.178		1.123		1.458	
	P	***		***		***		***		***		***		***	

***P<0.001; [abcgefghij] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.10 Fatty acid composition (% TFA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (second cut)

Population / Genotype		Fatty Acids (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	14.38 ghij	1.53 ab	1.33 hijk	1.76 abc	13.44 i	60.50 bc
	39	14.35 ghi	1.84 bcde	1.35 hijk	1.80 abc	12.86 hi	61.75 bcd
	115	15.06 ik	1.59 abc	1.60 l	2.18 c	13.51 i	59.85 b
	132	13.55 bcdefgh	1.66 abcde	1.46 k	1.91 abc	11.64 bcdefgh	64.13 defg
	134	14.43 hij	1.66 abcde	1.32 ghijk	1.79 abc	12.39 ghi	63.25 cdef
	148	13.81 cdefgh	2.01 efg	1.48 k	1.65 abc	11.50 bcdefgh	64.68 defg
	204	15.38 k	1.46 a	1.22 fghi	2.71 d	16.04 j	56.13 a
	213	14.07 efgh	1.77 abcde	1.26 fghi	1.66 abc	12.47 ghi	62.80 cde
	216	13.31 bcde	1.94 cde	0.97 abc	2.02 bc	10.40 bc	65.25 defg
	223	14.07 efgh	1.81 abcde	1.31 ghijk	1.40 a	12.02 cdefghi	63.47 cdefg
	231	14.24 fgh	1.73 abcde	1.28 fghij	1.71 abc	10.98 bcdefg	64.10 defg
	235	13.84 cdefgh	1.71 abcde	1.24 fghi	1.69 abc	10.56 bcde	65.42 defghi
	238	14.01 defgh	1.70 abcde	1.12 bdef	1.58 ab	11.28 bcdefgh	65.01 defg
	298	12.52 a	1.56 ab	1.17 defgh	1.70 abc	11.32 bcdefgh	66.63 fghijkl
	301	13.47 bcdefg	1.68 abcde	1.21 efghi	1.57 ab	10.57 bcdef	65.76 efghij
	307	14.32 ghi	1.71 abcde	1.27 fghij	1.82 abc	12.15 defghi	62.98 cdef
	314	13.33 abcdef	1.45 a	1.44 jk	2.05 bc	12.28 eghi	62.87 cdef
	320	13.69 cdefgh	1.61 abc	1.36 ijk	1.46 a	10.53 bcd	65.78 efghij
	329	13.40 bcdef	1.65 abcd	1.22 fghi	1.45 a	10.79 bcdefg	65.08 defg
	332	13.62 bcdefgh	1.59 abc	0.97 ab	1.85 abc	10.79 bcdefg	65.82 efghijk
Aurora x AberMagic	**81**	13.08 abc	2.01 ef	1.06 abcde	1.45 a	8.74 a	68.70 hjl
	86	12.78 ab	1.80 abcde	1.03 abcd	1.40 a	8.68 a	69.23 l
	103	13.15 abcd	1.97 de	1.14 defg	1.75 abc	11.15 bcdefg	65.39 efgh
	182	13.01 abc	2.26 f	0.93 a	1.76 abc	9.96 b	67.14 ghijkl
	s.e.d	0.269	0.103	0.055	0.154	0.502	1.058
	P	***	***	***	***	***	***

***P<0.001; [abcgefghijkl] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

lowest content of this FA (0.40 g kg^{-1} DM). The genotypes with the lowest and highest content of C18:0 were 182 and 148 with 0.30 and 0.48 g kg^{-1} DM, respectively, and also had the lowest and highest content of this FA in Cut 1. Genotype 204 also had the highest content of C18:1*cis*-9 and C18:2n-6 while having the lowest C18:3n-3 content (0.76, 4.46 and 15.70 g kg^{-1} DM, respectively). The lowest C18:1*cis*-9 (0.41 g kg^{-1} DM) and C18:2n-6 (3.18 g kg^{-1} DM) content was attributed to genotypes 223 and 81, respectively. Genotype 86 had the highest C18:3n-3 content at 27.17 g kg^{-1} DM. This genotype also had the highest TFA content (39.23 g kg^{-1} DM) while genotype 27 had the lowest TFA content (27.26 g kg^{-1} DM).

Overall mean individual FA proportions of Cut 2 were similar to Cut 1 proportion averages. Palmitic acid (C16:0) proportion ranged from 12.52 to 15.38% TFA with genotype 298 having the lowest proportion while genotype 204 had the highest proportion. Genotypes 314 to 182 were the lowest and highest for C16:1*trans*-3 proportion, ranging from 1.45 to 2.26% TFA. The lowest C18:0 proportion of 0.93% TFA was attributed to genotype 182 whereas genotype 148 had the highest proportion of this FA at 1.48% TFA. Genotype 204 had the highest proportions of C18:1*cis*-9 and C18:2n-6, at 2.71 and 16.04% TFA, respectively, while also having the lowest C18:3n-3 proportion of 56.13% TFA. The reverse was true for genotype 86 which had the lowest proportions of C18:1*cis*-9 and C18:2n-6 while having the highest proportion of C18:3n-3 (1.40, 8.68 and 69.23% TFA, respectively). However, genotype 223 had an equally low C18:1*cis*-9 proportion as genotype 86.

♦ *Cut 3 (Early August)*

The content and proportion of FAs for Cut 3 are presented in Table 5.11 and Table 5.12, respectively. Genotype differences were noted for both FA content and composition ($P<0.001$), and the ranking of the genotypes was different to Cuts 1 and 2. Overall average TFA content was 3.1% DM (31.01 g kg^{-1} DM), which is marginally lower than Cut 2. Genotype 314 had the lowest content of C16:0 and C16:1*trans*-3 while genotypes 86 and 103 had the highest content, respectively. The ranges of these FAs were 3.80 to 6.20 g kg^{-1} DM for C16:0 and 0.29 to 0.71 g kg^{-1} DM for C16:1*trans*-3. Genotype 332 had the lowest C18:0 content of 0.34 g kg^{-1} DM whereas genotypes 27 and 148 had the equally highest content of C18:0 (0.54 g kg^{-1} DM). Oleic acid (C18:1*cis*-9*)* content ranged from 0.48 to 0.82 g kg^{-1} DM, with genotypes 81 and 204 respectively having the lowest and highest content of this FA. The content of C18:2n-6, which ranged from 3.40 to 4.75 g kg^{-1} DM, was lowest in genotype 320 and highest in genotype 103. Both C18:3n-3 and TFA content were lowest in genotype 314 and highest in genotype 86, the ranges of which were 13.29 to 27.49 g kg^{-1} DM and 24.58 to 41.63 g kg^{-1} DM, respectively.

The ranges in proportion of C16:0, C16:1trans-3, C18:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3 were 13.41 to 16.89% TFA, 1.17 to 1.98% TFA, 1.00 to 1.94% TFA, 1.34 to 3.12% TFA, 9.58 to 16.91% TFA and 53.35 to 66.04% TFA, respectively. The corresponding lowest and highest FA proportions were genotype 298 and 115 for C16:0, 132 and 182 for C16:1*trans*-3, 332 and 27 for C18:0, 81 and 204 for C18:1*cis*-9, 86 and115 for C18:2n-6 and lastly, 27 and 86 for C18:3n-3.

Table 5.11 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (third cut)

Population / Genotype		Fatty Acid (g kg^{-1} DM)													
		C16:0		**C16:1t3**		**C18:0**		**C18:1c9**		**C18:2n-6**		**C18:3n-3**		**Total**	
B674G	**27**	4.41	abc	0.42	def	0.54	g	0.79	eg	4.55	def	14.84	abcd	27.79	abcde
	39	4.10	ab	0.40	bcde	0.40	abcde	0.66	bcd	4.08	abcdef	14.20	ab	25.71	ab
	115	4.70	bcde	0.39	bcde	0.46	def	0.69	def	4.66	ef	15.23	abcd	28.02	abcde
	132	4.41	bc	0.31	ab	0.48	f	0.65	abcd	4.08	abcdef	14.53	ab	26.22	abc
	134	4.51	bcd	0.41	def	0.40	abcde	0.49	ab	3.70	abc	16.66	abcde	27.99	abcde
	148	5.24	efg	0.54	g	0.54	g	0.61	abcd	4.07	abcdef	20.39	fgh	33.31	fghi
	204	4.26	abc	0.31	abc	0.40	abcde	0.82	g	3.87	abcd	14.67	abc	26.28	abc
	213	4.30	abc	0.38	abd	0.39	abc	0.67	cd	4.34	cdef	14.94	abcd	26.93	abc
	216	4.77	bcde	0.49	defg	0.37	ab	0.65	bcd	3.97	abcde	17.29	bcdef	29.59	bcdef
	223	4.83	cdef	0.44	defg	0.43	bcdef	0.55	abcd	4.25	bcdef	18.24	cdefg	31.02	cdefg
	231	4.65	bcde	0.48	defg	0.43	bcdef	0.61	abcd	3.83	abcd	17.32	bcdef	29.40	abcdef
	235	4.56	bcde	0.43	defg	0.40	abcde	0.50	abc	3.67	abc	17.71	bcdef	29.25	abcdef
	238	4.95	cdef	0.51	efg	0.44	bcdef	0.54	abcd	4.12	abcdef	19.28	efgh	32.04	defgh
	298	5.12	defg	0.63	h	0.45	cdef	0.64	abcd	4.66	ef	24.62	j	38.22	j
	301	5.10	defg	0.52	fg	0.47	ef	0.57	abcd	4.02	abcde	22.37	hij	35.19	ghij
	307	4.36	abc	0.43	defg	0.37	ab	0.52	abcd	4.01	abcde	15.76	abcd	27.49	abcd
	314	3.80	a	0.29	a	0.43	bcdef	0.63	abcd	3.99	abcde	13.29	a	24.58	a
	320	4.64	bcde	0.44	defg	0.45	cdef	0.51	abc	3.40	a	18.37	defg	29.76	bcdef
	329	4.90	cdef	0.51	efg	0.42	bcdef	0.56	abcd	4.15	abcdef	19.75	efgh	32.59	efghi
	332	5.14	defg	0.47	defg	0.34	a	0.57	abcd	3.94	abcde	22.08	hij	34.53	ghij
Aurora x AberMagic	**81**	5.47	fg	0.66	h	0.39	abcd	0.48	a	3.52	ab	23.68	ij	36.23	hij
	86	6.20	h	0.61	h	0.43	bcdef	0.58	abcd	4.00	abcde	27.49	k	41.63	k
	103	5.67	g	0.71	h	0.44	cdef	0.68	de	4.75	f	22.31	hij	36.78	ij
	182	5.21	efg	0.67	h	0.35	a	0.56	abcd	3.65	abc	21.38	ghi	33.76	fghi
	s.e.d	0.201		0.035		0.020		0.047		0.210		1.101		1.477	
	P	***		***		***		***		***		***		***	

***P<0.001; ^{abcgefghijk} Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.12 Fatty acid composition (% TFA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (third cut)

Population / Genotype		Fatty Acids (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	15.89 def	1.49 cd	1.94 n	2.83 ij	16.38 gh	53.35 a
	39	15.97 def	1.54 cd	1.57 ijk	2.57 hi	15.95 gh	55.09 abcd
	115	16.89 i	1.39 abcd	1.65 kl	2.51 ghi	16.91 h	53.88 ab
	132	16.80 gi	1.17 a	1.83 mn	2.48 ghi	15.58 gh	55.41 abcde
	134	16.13 fgh	1.47 cd	1.44 efghi	1.77 abcd	13.24 cdef	59.49 gh
	148	15.72 cdef	1.63 cde	1.61 jk	1.82 abcdef	12.23 bcde	61.21 hijk
	204	16.19 fgh	1.19 ab	1.54 hijk	3.12 j	14.70 fg	55.87 abcdef
	213	15.96 ef	1.43 bcd	1.43 efghi	2.47 hi	16.13 gh	55.47 abcde
	216	16.13 fg	1.67 de	1.26 cde	2.21 dfgh	13.43 cdef	58.43 defgh
	223	15.58 cdef	1.43 cd	1.37 defgh	1.77 abcde	13.68 cef	58.84 efgh
	231	15.83 def	1.63 cde	1.45 fghij	2.09 cdefg	13.07 cdef	58.82 fgh
	235	15.64 cdef	1.47 cd	1.39 defgh	1.71 abc	12.58 bcdef	60.45 ghij
	238	15.49 cdef	1.57 cde	1.38 defgh	1.69 abc	12.90 bcdef	60.05 ghi
	298	13.41 a	1.66 de	1.18 bc	1.68 abc	12.21 bcde	64.36 kl
	301	14.48 b	1.49 cd	1.34 cdefg	1.63 abc	11.44 abcd	63.57 ijkl
	307	15.84 def	1.58 cde	1.36 defgh	1.90 bcdef	14.55 fg	57.34 bdefg
	314	15.47 cdef	1.19 ab	1.77 lm	2.58 hi	16.31 gh	53.94 abc
	320	15.59 cdef	1.47 cd	1.50 ghijk	1.71 abc	11.43 abc	61.69 hijk
	329	15.05 bcd	1.56 cde	1.29 cdef	1.70 abc	12.79 bcdef	60.50 ghij
	332	14.89 bc	1.35 abc	1.00 a	1.65 abc	11.42 bc	63.89 jkl
Aurora x AberMagic	**81**	15.12 bcde	1.82 ef	1.09 ab	1.34 a	9.78 a	65.28 l
	86	14.89 bc	1.47 cd	1.04 a	1.40 ab	9.58 a	66.04 l
	103	15.41 cdef	1.92 f	1.21 bcd	1.86 bcdef	12.97 bcdef	60.58 ghij
	182	15.43 cdef	1.98 f	1.03 a	1.67 abc	10.83 ab	63.31 ijkl
	s.e.d	0.261	0.086	0.057	0.149	0.669	1.151
	P	***	***	***	***	***	***

***P<0.001; [abcgefghijklmn] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

♦ *Cut 4 (Late August)*

In terms of FA content, highly significant genotype differences were observed for C16:0, C18:0, C18:2n-6, C18:3n-3 and TFA ($P<0.001$) but were less apparent for C16:1*trans*-3 and C18:1*cis*-9 ($P<0.01$) (Table 5.13). Overall average TFA content increased to 3.4% DM (34.45 g kg^{-1} DM) relative to the earlier cuts (Cut 1, 2 and 3). The concentration of C16:0, C16:1*trans*-3 and C18:0 ranged from 4.05 to 5.68, 0.42 to 0.68 and 0.39 to 0.55 g kg^{-1} DM, respectively. Genotypes 314, 307 and 332 had the lowest content of these FAs while 86, 81 and 148 had the highest. Genotype 298 had the highest C18:1*cis*-9 and C18:2n-6 content, at 0.67 and 4.87 g kg^{-1} DM, respectively. The lowest concentrations of these FAs were attributed to genotype 329 (0.43 g kg^{-1} DM) for C18:1*cis*-9 content, and genotype 231 (3.52 g kg^{-1} DM) for C18:2n-6. Linolenic acid (C18:3n-3) ranged from 17.68 to 29.13 g kg^{-1} DM and TFA content ranged from 28.96 to 42.71 g kg^{-1} DM. Genotype 27 had the lowest C18:3n-3 and TFA content while genotype 86 had the highest content of these.

Genotype differences were also noted for proportion of C18:0, C18:2n-6 and C18:3n-3 ($P<0.001$), but less so for C16:1*trans*-3 ($P<0.01$) and no difference was found for C16:0 proportion (Table 5.14). Significant genotype differences were also noted for C18:1*cis*-9 ($P<0.001$), yet the multiple comparisons test failed to identify these differences, possibly due to groupings in the means (Thomas, 1973). The proportions of C16:1*trans*-3 and C18:1*cis*-9 ranged from 1.32 to 1.69% TFA and 1.31 to 1.86% TFA, respectively. Genotype 134 had the lowest proportions of these FAs while genotype 27 had the highest C16:1*trans*-3 and genotype 314 had the highest C18:1*cis*-9 proportion. The highest proportions of C18:0 and C18:2n-6 were attributed to genotype 132 (1.65% TFA and 13.44% TFA, respectively), which also had the

Table 5.13 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (fourth cut)

Population / Genotype		Fatty Acid (g kg^{-1} DM)						
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**
B674G	**27**	4.17 ^{ab}	0.49 ^{abc}	0.41 ^{a}	0.53 ^{abc}	3.57 ^{ab}	17.68 ^{a}	28.96 ^{a}
	39	4.38 ^{abc}	0.49 ^{abc}	0.43 ^{a}	0.54 ^{abc}	3.89 ^{ab}	19.67 ^{ab}	31.49 ^{abc}
	115	4.51 ^{abcd}	0.49 ^{abc}	0.48 ^{ab}	0.54 ^{abc}	4.03 ^{ab}	19.04 ^{ab}	31.27 ^{abc}
	132	4.33 ^{abc}	0.43 ^{ab}	0.49 ^{ab}	0.54 ^{abc}	3.97 ^{ab}	17.76 ^{a}	29.53 ^{ab}
	134	5.24 ^{abcd}	0.51 ^{abc}	0.46 ^{ab}	0.49 ^{abc}	4.04 ^{ab}	25.46 ^{abc}	38.26 ^{abc}
	148	4.94 ^{abcd}	0.50 ^{abc}	0.55 ^{b}	0.53 ^{abc}	3.89 ^{ab}	22.36 ^{abc}	35.00 ^{abc}
	204	4.83 ^{abcd}	0.55 ^{abc}	0.41 ^{a}	0.61 ^{abc}	3.92 ^{ab}	21.94 ^{abc}	34.54 ^{abc}
	213	4.66 ^{abcd}	0.49 ^{abc}	0.47 ^{ab}	0.59 ^{abc}	4.02 ^{ab}	20.15 ^{abc}	32.67 ^{abc}
	216	4.81 ^{abcd}	0.50 ^{abc}	0.47 ^{ab}	0.60 ^{abc}	4.02 ^{ab}	21.06 ^{abc}	33.69 ^{abc}
	223	4.63 ^{abcd}	0.49 ^{abc}	0.42 ^{a}	0.44 ^{ab}	3.79 ^{ab}	20.49 ^{abc}	32.61 ^{abc}
	231	4.24 ^{ab}	0.44 ^{abc}	0.46 ^{ab}	0.54 ^{abc}	3.52 ^{a}	18.77 ^{ab}	30.11 ^{ab}
	235	4.86 ^{abcd}	0.49 ^{abc}	0.47 ^{ab}	0.51 ^{abc}	3.73 ^{ab}	22.49 ^{abc}	34.84 ^{abc}
	238	4.62 ^{abcd}	0.50 ^{abc}	0.45 ^{ab}	0.47 ^{ab}	4.00 ^{ab}	21.36 ^{abc}	33.79 ^{abc}
	298	5.27 ^{abcd}	0.59 ^{abc}	0.54 ^{b}	0.67 ^{c}	4.87 ^{c}	24.88 ^{abc}	39.17 ^{abc}
	301	5.30 ^{bcd}	0.55 ^{abc}	0.48 ^{ab}	0.61 ^{abc}	4.28 ^{abc}	25.05 ^{abc}	38.70 ^{abc}
	307	4.24 ^{ab}	0.42 ^{a}	0.43 ^{a}	0.50 ^{abc}	3.71 ^{ab}	20.01 ^{abc}	31.58 ^{abc}
	314	4.05 ^{a}	0.46 ^{abc}	0.44 ^{a}	0.55 ^{abc}	3.83 ^{ab}	17.80 ^{a}	29.37 ^{ab}
	320	4.55 ^{abcd}	0.44 ^{abc}	0.47 ^{ab}	0.51 ^{abc}	3.54 ^{ab}	20.26 ^{abc}	31.85 ^{abc}
	329	4.42 ^{abc}	0.48 ^{abc}	0.41 ^{a}	0.43 ^{a}	3.65 ^{ab}	20.10 ^{abc}	31.61 ^{abc}
	332	4.95 ^{abcd}	0.48 ^{abc}	0.39 ^{a}	0.53 ^{abc}	3.84 ^{ab}	23.78 ^{abc}	36.32 ^{abc}
Aurora x AberMagic	**81**	5.53 ^{cd}	0.68 ^{c}	0.42 ^{a}	0.55 ^{abc}	3.79 ^{ab}	27.74 ^{bc}	41.05 ^{bc}
	86	5.68 ^{d}	0.64 ^{abc}	0.43 ^{a}	0.56 ^{abc}	3.91 ^{ab}	29.13 ^{c}	42.71 ^{c}
	103	5.27 ^{abcd}	0.63 ^{abc}	0.46 ^{ab}	0.63 ^{bc}	4.50 ^{bc}	24.34 ^{abc}	38.18 ^{abc}
	182	5.49 ^{cd}	0.67 ^{bc}	0.43 ^{a}	0.61 ^{abc}	4.18 ^{ab}	25.79 ^{abc}	39.44 ^{abc}
	s.e.d	0.334	0.067	0.030	0.050	0.260	2.578	3.198
	P	***	**	***	**	***	***	***

**P<0.01; ***P<0.001; ^{abcd} Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.14 Fatty acid composition (% TFA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (fourth cut)

Population / Genotype		Fatty Acids (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	14.43	1.69 b	1.42 abcde	1.83 a	12.37 abc	60.89 a
	39	13.93	1.56 ab	1.38 abcde	1.71 a	12.43 bc	62.30 abc
	115	14.49	1.57 ab	1.54 cde	1.75 a	12.99 c	60.63 a
	132	14.67	1.45 ab	1.65 e	1.84 a	13.44 c	60.12 a
	134	13.80	1.32 a	1.24 abcde	1.31 a	10.85 abc	66.02 abc
	148	14.11	1.43 ab	1.57 de	1.51 a	11.10 abc	63.92 abc
	204	14.01	1.57 ab	1.19 abcd	1.82 a	11.54 abc	63.25 abc
	213	14.30	1.52 ab	1.45 bcde	1.81 a	12.35 bc	61.53 ab
	216	14.34	1.49 ab	1.41 abcde	1.77 a	12.02 abc	62.35 abc
	223	14.22	1.50 ab	1.30 abcde	1.36 a	11.65 abc	62.79 abc
	231	14.07	1.45 ab	1.52 cde	1.80 a	11.80 abc	62.12 abc
	235	14.00	1.40 ab	1.36 abcde	1.48 a	10.79 abc	64.32 abc
	238	13.74	1.49 ab	1.32 abcde	1.38 a	11.78 abc	63.16 abc
	298	13.58	1.50 ab	1.42 abcde	1.72 a	12.56 bc	63.21 abc
	301	13.71	1.43 ab	1.25 abcde	1.57 a	11.03 abc	64.74 abc
	307	13.45	1.34 ab	1.35 abcde	1.60 a	11.77 abc	63.38 abc
	314	13.81	1.55 ab	1.50 cde	1.86 a	13.10 c	60.53 a
	320	14.30	1.40 ab	1.48 bcde	1.61 a	11.13 abc	63.55 abc
	329	13.99	1.53 ab	1.30 abcde	1.35 a	11.54 abc	63.59 abc
	332	13.64	1.34 ab	1.09 abc	1.45 a	10.65 abc	65.31 abc
Aurora x AberMagic	**81**	13.53	1.67 ab	1.04 ab	1.37 a	9.37 ab	67.29 bc
	86	13.32	1.50 ab	1.00 a	1.32 a	9.18 a	68.09 c
	103	13.91	1.64 ab	1.24 abcde	1.66 a	11.88 abc	63.51 abc
	182	14.04	1.68 ab	1.14 abcd	1.58 a	10.88 abc	64.81 abc
	s.e.d	0.415	0.097	0.121	0.149	0.872	1.669
	P	NS	**	***	***	***	***

NS, Not significant; **P<0.01; ***P<0.001; [abcde] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

lowest C18:3n-3 proportion (60.12% TFA). The opposite of this was true for genotype 86, which had the lowest proportions of C18:0 and C18:2n-6 while having the highest proportion of C18:3n-3 (1.00, 9.18 and 68.09% TFA, respectively).

♦ *Cut 5 (Late September)*

Genotype differences were observed for both FA content and proportions ($P<0.001$; Table 5.15 and Table 5.16), apart from C18:2n-6 content where genotype differences were smaller ($P<0.01$). Overall average TFA content for Cut 5 was 4.2% DM (41.94 g kg^{-1} DM), which was the highest of all cuts. Palmitic acid (C16:0) content ranged from 4.87 to 6.34 g kg^{-1} DM, with genotype 314 having the lowest content and genotype 86 having the highest. The lowest C16:1*trans*-3 content (0.62 g kg^{-1} DM) was attributed to genotype 307 while genotype 182 had the highest content (1.11 g kg^{-1} DM). This genotype also had the lowest C18:0 content of 0.48 g kg^{-1} DM whereas genotype 148 had the highest C18:0 content at 0.79 g kg^{-1} DM. The range in C18:1*cis*-9 content was 0.50 to 0.92 g kg^{-1} DM, with genotypes 329 and 103 having the lowest and highest content, respectively. Linoleic acid (C18:2n-6) content was lowest in genotype 27 at 4.31 g kg^{-1} DM and highest in genotype 298 at 5.52 g kg^{-1} DM. Genotype 86 had the highest C18:3n-3 and TFA content at 34.39 and 50.47 g kg^{-1} DM, respectively. However, the lowest content of C18:3n-3 (22.14g kg^{-1} DM) was attributed to genotype 307 and the lowest TFA content (36.31g kg^{-1} DM) to genotype 39.

Table 5.15 Fatty acid content (g kg^{-1} DM) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (fifth cut)

Population / Genotype		Fatty Acid (g kg^{-1} DM)													
		C16:0		**C16:1t3**		**C18:0**		**C18:1c9**		**C18:2n-6**		**C18:3n-3**		**Total**	
B674G	**27**	5.11	abcd	0.73	ab	0.53	abc	0.59	abcde	4.31	a	24.76	ab	38.42	ab
	39	4.98	ab	0.66	a	0.57	abcde	0.62	abcdef	4.84	ab	22.32	a	36.31	a
	115	5.81	abcde	0.82	ab	0.66	ef	0.67	bcdefg	5.10	ab	26.62	abcd	42.09	abcdef
	132	5.69	abcde	0.73	ab	0.68	f	0.65	bcdef	5.28	ab	25.07	ab	40.25	abcd
	134	5.68	abcde	0.65	a	0.61	cdef	0.60	abcde	5.02	ab	26.80	abcd	41.63	abcdef
	148	5.55	abcde	0.66	a	0.79	g	0.69	cdefg	4.69	ab	25.62	abc	40.05	abc
	204	5.36	abcde	0.66	a	0.50	ab	0.65	bcdef	4.94	ab	23.02	a	37.72	ab
	213	5.16	abcd	0.74	ab	0.53	abc	0.59	abcde	5.16	ab	23.10	a	37.60	ab
	216	5.89	abcde	0.90	bcd	0.55	abc	0.71	defg	5.14	ab	27.95	abcde	43.89	abcdefg
	223	5.61	abcde	0.75	ab	0.58	bcde	0.53	ab	4.62	ab	25.61	abc	40.32	abcde
	231	5.42	abcde	0.75	ab	0.57	abcde	0.61	abcde	4.56	ab	24.96	ab	39.09	abc
	235	5.83	abcde	0.73	ab	0.65	def	0.58	abcde	5.11	ab	26.40	abcd	41.83	abcdef
	238	5.58	abcde	0.77	ab	0.55	abc	0.56	abc	4.93	ab	27.08	abcd	41.86	abcdef
	298	5.47	abcde	0.87	bc	0.57	abcde	0.76	fg	5.52	b	29.70	bcdef	45.29	bcdefg
	301	5.86	abcde	0.75	ab	0.59	bcde	0.65	bcdef	4.93	ab	29.56	bcdef	44.78	abcdefg
	307	5.06	abc	0.62	a	0.60	bcdef	0.63	abcdef	5.14	ab	22.14	a	36.58	a
	314	4.87	a	0.66	a	0.57	abcde	0.70	cdefg	4.79	ab	22.71	a	36.79	ab
	320	5.46	abcde	0.72	ab	0.65	def	0.57	abcd	4.91	ab	24.60	ab	39.09	abc
	329	5.19	abcd	0.81	ab	0.52	abc	0.50	a	4.54	ab	24.99	ab	39.23	abc
	332	6.03	cde	0.80	ab	0.56	abcd	0.68	cdefg	5.12	ab	31.07	cdef	46.80	cdefg
Aurora x AberMagic	**81**	6.10	cde	1.09	e	0.51	abc	0.67	cdefg	4.59	ab	34.27	f	49.73	g
	86	6.34	e	0.98	cde	0.56	abcd	0.79	g	4.83	ab	34.39	f	50.47	g
	103	6.09	de	1.04	de	0.60	bcdef	0.92	h	5.42	b	31.84	def	48.51	fg
	182	5.91	bcde	1.11	e	0.48	a	0.72	efg	4.70	ab	32.79	ef	48.13	dfg
	s.e.d	0.280		0.056		0.028		0.041		0.283		1.770		2.378	
	P	***		***		***		***		**		***		***	

**P<0.01; ***P<0.001; [abcdefgh] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Table 5.16 Fatty acid composition (% TFA) of different genotypes of perennial ryegrass (*Lolium perenne*) from two distinct populations (fifth cut)

Population / Genotype		Fatty Acids (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	13.28 cdef	1.90 bcde	1.38 cdefg	1.54 bcdefgh	11.23 b	64.46 cde
	39	13.73 def	1.82 bcd	1.59 hi	1.72 fghi	13.39 dfg	61.31 abc
	115	13.85 def	1.94 bcde	1.57 hi	1.59 cdefgh	12.18 bcdef	63.11 abcde
	132	14.16 ef	1.81 abcd	1.69 i	1.63 defgh	13.14 cdefg	62.23 abcd
	134	13.69 def	1.56 a	1.46 fgh	1.44 abcde	12.08 bcdef	64.25 bcde
	148	13.86 def	1.66 ab	1.97 j	1.71 fghi	11.70 bcde	63.97 bcde
	204	14.25 f	1.74 abc	1.33 cdef	1.74 hij	13.20 cdefg	60.83 ab
	213	13.75 def	1.97 cde	1.41 defgh	1.56 bcdefgh	13.71 fg	61.36 abc
	216	13.41 cdef	2.05 def	1.25 bcde	1.63 defgh	11.70 bcd	63.67 abcde
	223	13.95 def	1.86 bcd	1.44 efgh	1.31 ab	11.48 bc	63.47 abcde
	231	13.88 def	1.91 bcde	1.45 fgh	1.55 bcdefgh	11.68 bcd	63.77 abcde
	235	13.95 def	1.74 abc	1.55 ghi	1.39 abcd	12.21 bcdef	63.10 abcde
	238	13.33 cdef	1.84 bcd	1.31 cdef	1.33 abc	11.73 bcde	64.74 cde
	298	12.07 a	1.93 bcde	1.26 bcde	1.67 efgh	12.15 bcdef	65.59 def
	301	13.08 bcde	1.66 ab	1.32 cdef	1.46 abcdef	11.01 b	66.02 ef
	307	13.85 def	1.68 ab	1.64 i	1.73 fhi	14.14 g	60.35 a
	314	13.27 cdef	1.80 abcd	1.56 ghi	1.91 ij	13.06 cdefg	61.61 abc
	320	13.96 def	1.84 bcd	1.66 i	1.46 abcdefg	12.55 bcdef	62.92 abcde
	329	13.27 cdef	2.07 def	1.33 cdef	1.28 a	11.58 bc	63.63 abcde
	332	12.89 abcd	1.70 abc	1.19 bc	1.46 abcdef	10.96 b	66.35 efg
Aurora x AberMagic	**81**	12.25 ab	2.21 fg	1.02 a	1.35 abc	9.21 a	68.93 g
	86	12.57 abc	1.94 bcde	1.11 ab	1.57 cdefgh	9.58 a	68.13 fg
	103	12.57 abc	2.13 efg	1.24 bcd	1.89 i	11.18 b	65.60 def
	182	12.29 ab	2.29 g	0.99 a	1.50 abcdefgh	9.78 a	68.11 fg
	s.e.d	0.310	0.078	0.059	0.076	0.502	1.013
	P	***	***	***	***	***	***

***P<0.001; [abcdefghij] Values not sharing common superscripts within columns differ significantly (P<0.05) according to Student-Newman-Keuls multiple comparisons test.

Proportion of C16:0 ranged from 12.07 to 14.25% TFA with genotypes 298 and 204 respectively having the lowest and highest proportion of this FA. Genotype 182 had the highest proportion of C16:1*trans*-3 (2.29% TFA) and the lowest proportion of C18:0 (0.99% TFA). The lowest C16:1*trans*-3 proportion of 1.56% TFA was attributed to genotype 134 whereas genotype 148 had the highest proportion of C18:0 at 1.97% TFA. Oleic acid (C18:1 *cis*-9) proportion was lowest in genotype 329 and highest in genotype 314 (1.28 and 1.91% TFA, respectively). The proportions of C18:2n-6 and C18:3n-3 ranged from 9.21 to 14.14% TFA and 60.35 to 68.93, respectively. Genotype 81 had the lowest C18:2n-6 proportion and had the highest C18:3n-3 proportion whereas the inverse was true for genotype 307.

5.4.2.2 Genotype Performance across All Cuts

The overall FA content and proportion means for each genotype across all five cuts are presented in Table 5.17. Genotypes from the Aurora x AberMagic population had the highest overall mean content of C16:0, C16:1*trans*-3, C18:1*cis*-9, C18:3n-3 and TFA and lowest overall mean content of C18:0 and C18:2n-6, while the inverse was true for genotypes from the B674G population. Table 5.18 shows FA content and proportion averages at each cut across all twenty-four genotypes. Total and individual FA content generally increased from Cut 1 through to Cut 5. However, proportion of C16:0, C18:0, C18:1*cis*-9 and C18:2n-6 were highest at Cut 3. This cut also resulted in the lowest overall C18:3n-3 proportion. A summary of the output from the repeated measures ANOVA analysis, which took into account both genotype and cut along with their interaction, is given in Table 5.19. This analysis found highly significant effects of both genotype and cut in addition to highly significant genotype x cut interaction for all individual and total FA contents and all individual FA proportions.

Concerning the genotype means across all five cuts (Table 5.17), genotype 314 had the lowest overall mean content of C16:0 and C16:1*trans*-3 (4.08 and 0.45 g kg^{-1} DM, respectively). The highest overall mean content of 5.51 g kg^{-1} DM for C16:0 was attributed to genotype 86 while genotype 81 had the highest overall mean C16:1*trans*-3 content (0.79 g kg^{-1} DM). Stearic acid (C18:0), C18:1*cis*-9 and C18:2n-6 overall average content ranged from 0.36 to 0.56 g kg^{-1} DM, 0.48 to 0.70 g kg^{-1} DM and 3.62 to 4.58 g kg^{-1} DM, respectively. Genotype 182, 223 and 81 had the lowest content of these FAs while genotype 148, 103 and 298 had the highest content of these FAs. Genotype 314 had the lowest overall average C18:3n-3 and TFA content, at 17.83 and 29.58 g kg^{-1} DM, respectively. The highest C18:3n-3 content of 28.34 g kg^{-1} DM and TFA content of 41.76 g kg^{-1} DM were attributed to genotype 86.

With regard to FA proportions, C16:0 ranged from 12.78 to 14.95% TFA with the respective lowest and highest genotype being 298 and 115, both from the B674G population. Two genotypes (132 and 314) had equally low C16:1*trans*-3 proportion of 1.50% TFA while genotype 182 had the highest C16:1*trans*-3 proportion of 2.08% TFA. Stearic acid (C18:0) proportion ranged from 0.99 to 1.61% TFA with genotypes 182 and 148 having the respective lowest and highest proportion of this FA. Genotype 81 had the lowest proportion of C18:1*cis*-9 and C18:2n-6, at 1.41 and 9.12% TFA respectively. The genotypes with the highest proportion of these FAs were genotype 204 for C18:1*cis*-9 (2.24% TFA) and genotype 115 for C18:2n-6 (13.71% TFA). Genotype 115 also had the lowest C18:3n-3 proportion at 59.38% TFA while genotype 86 had the highest proportion at 67.83% TFA.

Table 5.17 Fatty acid content (g kg^{-1} DM) and proportion (% TFA) means for each genotype across all five cuts

Population		**Fatty Acid content (g kg^{-1} DM)**							**Fatty acid proportion (% TFA)**					
/ Genotype		**C16:0**	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**	**C16:0**	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
B674G	**27**	4.25	0.50	0.44	0.57	3.87	18.07	29.86	14.34	1.64	1.48	1.95	13.14	60.05
	39	4.32	0.53	0.44	0.57	4.02	18.51	30.39	14.31	1.73	1.44	1.93	13.36	60.56
	115	4.63	0.51	0.48	0.63	4.22	18.78	31.34	14.95	1.60	1.54	2.06	13.71	59.38
	132	4.51	0.47	0.49	0.62	4.06	18.78	30.82	14.74	1.50	1.59	2.06	13.26	60.61
	134	4.73	0.50	0.45	0.51	3.91	21.09	33.10	14.43	1.51	1.35	1.59	11.92	63.28
	148	4.84	0.60	0.56	0.57	3.94	21.76	34.24	14.14	1.76	1.61	1.66	11.47	63.53
	204	4.58	0.48	0.40	0.68	4.20	18.71	31.25	14.75	1.52	1.29	2.24	13.68	59.44
	213	4.52	0.54	0.43	0.58	4.20	19.38	31.80	14.28	1.68	1.36	1.84	13.30	60.71
	216	4.73	0.61	0.39	0.65	3.92	21.04	33.57	14.18	1.79	1.16	1.99	11.70	62.47
	223	4.61	0.55	0.43	0.48	3.88	20.20	32.36	14.28	1.69	1.33	1.50	12.03	62.35
	231	4.50	0.53	0.43	0.56	3.63	19.70	31.38	14.38	1.68	1.37	1.79	11.59	62.63
	235	4.68	0.52	0.45	0.52	3.77	20.87	32.95	14.26	1.55	1.35	1.62	11.45	63.16
	238	4.76	0.56	0.43	0.52	4.04	21.54	34.05	14.06	1.64	1.26	1.54	11.86	63.16
	298	4.96	0.66	0.48	0.66	4.58	25.42	38.97	12.78	1.68	1.23	1.69	11.75	65.15
	301	4.87	0.56	0.45	0.58	3.91	23.15	35.67	13.69	1.58	1.25	1.65	10.92	64.79
	307	4.36	0.49	0.42	0.55	3.95	18.94	30.78	14.23	1.59	1.36	1.79	12.84	61.47
	314	4.08	0.45	0.45	0.63	3.96	17.83	29.58	13.90	1.50	1.52	2.17	13.55	59.88
	320	4.54	0.51	0.47	0.51	3.64	20.11	31.75	14.33	1.60	1.46	1.63	11.42	63.29
	329	4.39	0.53	0.40	0.49	3.71	19.86	31.57	13.94	1.65	1.24	1.59	11.79	62.81
	332	4.82	0.54	0.37	0.57	3.83	23.31	35.56	13.57	1.52	1.04	1.61	10.74	65.47
Aurora x AberMagic	**81**	5.25	0.79	0.41	0.56	3.62	26.99	39.81	13.27	1.98	1.04	1.41	9.12	67.63
	86	5.51	0.73	0.43	0.62	3.87	28.34	41.76	13.20	1.74	1.03	1.49	9.24	67.83
	103	5.18	0.78	0.45	0.70	4.42	25.07	38.86	13.39	1.99	1.17	1.80	11.39	64.40
	182	4.85	0.76	0.36	0.60	3.66	23.98	36.28	13.45	2.08	0.99	1.68	10.14	65.82

Table 5.18 Fatty acid content (g kg^{-1} DM) and proportion (% TFA) means for each cut across all genotypes

Cut	Fatty Acid content (g kg^{-1} DM)							Fatty acid proportion (% TFA)					
	C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**	**C16:0**	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
1	3.89	0.51	0.34	0.54	3.20	18.45	28.99	13.47	1.74	1.17	1.88	11.13	63.41
2	4.35	0.55	0.39	0.55	3.61	20.45	31.72	13.79	1.74	1.24	1.76	11.50	64.24
3	4.82	0.48	0.43	0.61	4.08	18.69	31.16	15.56	1.53	1.40	2.01	13.36	59.46
4	4.79	0.52	0.45	0.55	3.94	21.97	34.46	13.98	1.50	1.34	1.60	11.59	63.39
5	5.59	0.79	0.58	0.65	4.92	26.98	41.94	13.38	1.88	1.40	1.56	11.86	64.06

Table 5.19 Summary of repeated measures ANOVA output for genotype, cut and genotype x cut interaction

		Fatty Acid content (g kg^{-1} DM)							Fatty acid proportion (% TFA)					
		C16:0	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**	**Total**	**C16:0**	**C16:1t3**	**C18:0**	**C18:1c9**	**C18:2n-6**	**C18:3n-3**
Genotype	**s.e.d.**	0.140	0.025	0.014	0.025	0.140	0.880	1.180	0.152	0.054	0.044	0.060	0.317	0.599
	P	***	***	***	***	***	***	***	***	***	***	***	***	***
Cut	**s.e.d.**	0.047	0.010	0.005	0.009	0.043	0.324	0.414	0.065	0.017	0.014	0.027	0.122	0.235
	P	***	***	***	***	***	***	***	***	***	***	***	***	***
Genotype x Cut	**s.e.d.**	0.250	0.049	0.025	0.047	0.236	1.669	2.162	0.323	0.091	0.076	0.132	0.622	1.192
	P	***	***	***	***	***	***	***	***	***	***	***	***	***

***P<0.001

As for individual cut averages across all genotypes, the general trend for FA content was to increase from Cut 1 through to Cut 5, whereas trends for FA proportion were more complicated. Palmitic acid (C16:0) increased steadily from 3.89 g kg^{-1} DM at Cut 1 to 5.59 g kg^{-1} DM at Cut 5, with a slight dip at Cut 4. The one FA which did not follow the trend was C16:1*trans*-3. The cut average content of this FA was similar for Cuts 1, 2, 3 and 4, with Cut 3 resulting in the lowest concentration (0.48 g kg^{-1} DM), while Cut 5 resulted in a markedly higher C16:1*trans*-3 content (0.79 g kg^{-1} DM). For the remaining individual FAs and for TFA, Cut 1 resulted in the lowest cut average content while Cut 5 resulted in the highest. The content of C18:0 was the only FA to continuously increase with successive cuts, with an average of 0.34 g kg^{-1} DM for the Cut 1 increasing to 0.58 g kg^{-1} DM for Cut 5. Content of C18:1*cis*-9 ranged from 0.54 to 0.65 g kg^{-1} DM. Cuts 1, 2 and 4 resulted in similar average content while Cuts 3 and 5 resulted in higher but similar content. Content of C18:2n-6 followed the same pattern as C18:1*cis*-9 between cuts, with Cuts 1, 2 and 4 being lower while Cuts 3 and 5 being higher. Linoleic acid (C18:2n-6) content averaged at 3.20 g kg^{-1} DM for Cut 1 increasing to 4.92 g kg^{-1} DM for Cut 5, while C18:3n-3 content ranged from 18.45 to 26.98 g kg^{-1} DM. Cuts 1 and 3 had very similar overall average C18:3n-3 content. Cuts 2 and 4 also had similar but slightly higher C18:3n-3 content whereas Cut 5 resulted in a noticeably higher C18:3n-3 average content. Total FA increased from 28.99 g kg^{-1} DM at Cut 1 to 41.94 g kg^{-1} DM at Cut 5, with a very minor decrease at Cut 3.

Proportions of C16:0, C18:0, C18:1*cis*-9 and C18:2n-6 were highest during Cut 3, which also resulted in the lowest C18:3n-3 proportion (15.56, 1.40, 2.01, 13.36 and 59.46% TFA, respectively). However, C18:0 average proportion was equally high at Cut 5 which also had the highest average C16:1*trans*-3 proportion of 1.88% TFA. Cuts

1, 2, 4 and 5 had similar average C16:0 proportion of between 13 and 14% TFA, while Cut 3 had a higher C16:0 proportion of 15.56% TFA. Average proportion for C16:1*trans*-3 was the same at Cut 1 and 2 (1.74% TFA). This decreased to 1.50% TFA by Cut 4 then increased to 1.88% TFA at Cut 5. Stearic acid (C18:0) average proportion was 1.17% TFA at Cut 1, increased to 1.40% TFA by Cut 3 and decreased to 1.34% TFA at Cut 4 before increasing to 1.40% at Cut 5. There was no clear trend between cuts in proportion of C18:1*cis*-9. The proportion of this FA was 1.88% TFA at Cut 1, then decreased to 1.76% TFA at Cut 2. Cut 3 resulted in a sizeable increase in C18:1*cis*-9 proportion to 2.01% TFA which then decreased to 1.60 and 1.56% TFA at Cuts 4 and 5, respectively. Proportion of C18:2n-6 was similar for Cuts 1, 2, 4 and 5 at approximately 11% TFA whereas Cut 3 resulted in an increase to 13.36% TFA. Linolenic acid (C18:3n-3) average proportion was also similar for Cuts 1, 2, 4 and 5 at between 63 and 64% TFA whereas Cut 3 resulted in a decrease in C18:3n-3 proportion to 59.46% TFA.

5.4.3 Chlorophyll vs. Fatty Acid Content

Positive correlations were found between FAs and both methods of chlorophyll quantification ($P<0.001$), however all FAs correlated better with the chlorophyll extraction method (*in vitro*) in comparison to the chlorophyll meter (*SPAD; in vivo*) method (see Table 5.20). Stronger correlations were found for both methods of chlorophyll quantification with C16:0, C18:3n-3 and TFA compared to C18:0, C18:1*cis*-9 and C18:2n-6. Interestingly, C16:1*trans*-3 had a high correlation with the chlorophyll extraction method ($r = 0.72$), yet a low correlation was found between this FA and the chlorophyll meter (*SPAD*) method ($r = 0.29$).

Table 5.20 Spearman's Rank correlations for *SPAD* and Chlorophyll vs. individual and total fatty acid content across all cuts (449 d.f.)

	Chlorophyll meter (SPAD)	*Chlorophyll extraction*
C16:0	**0.58**	**0.79**
C16:1trans-3	**0.29**	**0.72**
C18:0	**0.28**	**0.53**
C18:1cis-9	**0.24**	**0.34**
C18:2n-6	**0.38**	**0.52**
C18:3n-3	**0.43**	**0.87**
Total	**0.47**	**0.86**

NB: **Bold** P<0.001

Observing the data at a per cut level revealed some variability between cuts in the correlations of some of the FAs with either method of chlorophyll quantification (Table 5.21). Again, low to negligible correlations were generally found at each cut for both chlorophyll methods with C18:0, C18:1*cis*-9 and C18:2n-6. Conflicting results were found for the negligible correlations between C18:0 and the chlorophyll meter (*SPAD*) method, where a positive correlation existed when analysing the dataset regardless of cut ($r = 0.28$), but some significant negative correlations where found when analysing the data per cut ($r \leq -0.21$; $P<0.05$). Significantly negative correlations were also found for C18:2n-6 and the chlorophyll meter (*SPAD*) at Cuts 1 and 2 ($r \leq -0.23$; $P<0.05$). Moderate correlations were found for C16:0, C16:1*trans*-3, C18:3n-3 and TFA with the chlorophyll meter (*SPAD*) method for Cuts 2, 4 and 5 ($P<0.01$). However, noticeable weaker correlations between these FAs and chlorophyll method occurred at Cut 1 and 3, with these correlations becoming negligible at these sampling points ($P>0.05$). Conversely, the correlations for C16:0, C16:1*trans*-3, C18:3n-3 and TFA with chlorophyll extraction were consistently high at all cuts ($P<0.001$), with a slight dip at Cut 4.

Table 5.21 Spearman's Rank correlation of *SPAD* and chlorophyll vs. individual and total fatty acid content per cut (minimum 72 d.f.)

		Chlorophyll meter (SPAD)	*Chlorophyll extraction*
C16:0	*Cut 1*	-0.01	**0.59**
	Cut 2	**0.29**	**0.59**
	Cut 3	0.13	**0.81**
	Cut 4	**0.46**	**0.69**
	Cut 5	**0.49**	**0.73**
C16:1trans-3	*Cut 1*	0.16	**0.68**
	Cut 2	**0.34**	**0.58**
	Cut 3	0.17	**0.77**
	Cut 4	**0.27**	**0.49**
	Cut 5	**0.49**	**0.72**
C18:0	*Cut 1*	**-0.41**	0.19
	Cut 2	**-0.25**	0.06
	Cut 3	**-0.24**	0.18
	Cut 4	-0.15	0.08
	Cut 5	**-0.21**	-0.05
C181cis-9	*Cut 1*	0.11	0.15
	Cut 2	0.12	**0.21**
	Cut 3	0.07	-0.01
	Cut 4	0.08	**0.27**
	Cut 5	**0.24**	**0.52**
C18:2n-6	*Cut 1*	**-0.23**	0.16
	Cut 2	**-0.25**	-0.16
	Cut 3	-0.18	0.03
	Cut 4	0.10	**0.35**
	Cut 5	0.00	0.17
C18:3n-3	*Cut 1*	0.10	**0.77**
	Cut 2	**0.43**	**0.85**
	Cut 3	0.13	**0.94**
	Cut 4	**0.46**	**0.66**
	Cut 5	**0.64**	**0.90**
Total	*Cut 1*	0.05	**0.74**
	Cut 2	**0.37**	**0.79**
	Cut 3	0.11	**0.91**
	Cut 4	**0.45**	**0.66**
	Cut 5	**0.61**	**0.87**

NB: **Bold** P<0.05; **Bold** P<0.01; **Bold** P<0.001

5.5 Discussion

5.5.1 Chlorophyll Content

Olszewska *et al* (2008a, 2008b) found *SPAD* values of perennial ryegrass (*Lolium perenne*) and orchard grass (*Dactylis glomerata* L.), kept under glasshouse condition, generally increased over the growing period. The *SPAD* values ranged from 35.43 to 48.10 for control treatment plants within these studies, whereas a wider range was found in the present study (26.7 to 46.9). Similar trends were also found by Gabornik (2003) for plants maintained under field conditions, who reported values of 42.3, 44.3 and 44.0 for May, September and October harvests of *Lolium perenne* (cv. Mustang). These results are slightly above the average values found in the present study, which could be a result of differing climatic and/or environmental conditions.

Total chlorophyll content is more commonly reported in terms of fresh weight rather than dry weight. Nevertheless, Wang and Schjoerring (2012) included chlorophyll determination in their investigation into seasonality of ^{15}N and ^{13}C in grassland, which involved a similar sampling regime to the present study. They reported a decrease in total chlorophyll content between June and July followed by a slight but steady increase towards September, whereas the chlorophyll content steadily increased across all months between early June and late September in the present study. Kingston-Smith *et al* (2002) reported much more variable results during these months with a noticeable increase between mid-June and mid-July, followed by a sharp decrease in August and a slight increase in September.

Moderately positive correlations were found when comparing the non-destructive chlorophyll meter (*SPAD*) data with the *in vitro* chlorophyll data, with the exception of the first and third harvests, where correlations were negligible. Few studies have

gone to the effort of quantifying and validating the relationship between *SPAD* output and *in vitro* chlorophyll content (Uddling *et al.*, 2007). The studies which have performed such calibrations frequently define this relationship as linear, in accordance with Beer's law (which assumes that absorbance is solely dependent on pigment concentration). Dwyer *et al* (1991), Monje and Bugbee (1992), Xu *et al* (2000) and Ling *et al* (2011) reported R^2 values of 0.83, 0.98, 0.91 and 0.93 for corn, *Arabidopsis thaliana*, sorghum and grouped wheat, rice and soya bean data, respectively. However, other factors such as light scattering, leaf surface reflectivity and pigment spatial distribution can also influence light transmission and absorbance in leaves (Vogelmann, 1989). Consequently, some studies have reported slightly improved fit of this relationship through using polynomial or exponential functions and again reported high R^2 values (Monje and Bugbee, 1992; Markwell *et al.*, 1995; Bindi *et al.*, 2002; Uddling *et al.*, 2007; Ling *et al.*, 2011).

Environmental factors can have a large effect on the *in vivo*/*in vitro* chlorophyll relationship (Campbell *et al.*, 1990), which may explain why the correlation coefficients found in the present study were much lower than those reported above, with high variability between cuts. Also, many of the studies which have defined this relationship have involved plants with a larger leaf area compared to perennial ryegrass. The *SPAD*-502 meter has a 3 x 2 mm window, which must be completely covered by the leaf in order to gain an accurate reading. Due to the shape and size of perennial ryegrass leaves, *SPAD* measurements were often taken across the whole leaf width, thus including the mid-rib within the 3 x 2mm window, which may have increased the variability between individual measurements as this part of the leaf is thicker. Also, a delay to the start of the growing season because of cold temperatures during March and relatively dry conditions during June and July meant that many

plants had under-developed leaves at Cut 1 and Cut 3. This limited and added some bias into collecting twenty representative *SPAD* measurements per plant during these harvests because many of the leaves were too small to be measured accurately.

5.5.2 Fatty Acid Content and Composition

Fatty acid content increased during the growing season, with approximately a 1.5-fold increase for individual and TFA content between Cut 1 and Cut 5. Several previous studies have investigated the effects of vegetative stage/cutting date on forage FAs, however, as Glasser *et al* (2013) explains, inconsistencies in experimental design of these studies make it difficult to openly compare results. Experiment 1 of Dewhurst *et al* (2002) is of similar design to the present study wherein samples of perennial ryegrass were collected on average every 26 days from May to November. However, they reported lower TFA and C18:3n-3 content compared to the present study. This could be due to a number of reasons, such as differences in climatic conditions or inclusion of stem in the sample analysed. The present study determined FA content on purely leaf material from single plants, whereas earlier studies have sampled whole plants or plots which, in some instances, included stemmy material as well as leaf material. Leaf to stem ratio is known to affect the FA content of forages (Hawke, 1973). This difference in sample composition also likely explains why no decrease in TFA content is observed in the present study during the normal heading period.

Overall average proportions of individual FAs were fairly consistent between cuts, with the exception of Cut 3, where a decrease in C18:3n-3 and C16:1*trans*-3 proportion was observed while the proportion of the other major FAs increased. Two months of moderate precipitation leading up to Cut 3 harvest resulted in the plants undergoing borderline drought-stress at the time of Cut 3 harvest, which is the probable cause of this shift in FA proportions. Decreases in C18:3n-3 proportion in response to drought

stress has been demonstrated in other plant species such as cotton (Ferrari-Iliou *et al.*, 1984) and rape (Dakhma *et al.*, 1995).

Differences in FA content and composition between different plant families, species and cultivars have previously been reported (Dewhurst *et al.*, 2001, 2002; Gilliland *et al.*, 2002; Boufaïed *et al.*, 2003; Elgersma *et al.*, 2003b). For instance, Dewhurst *et al* (2001) reported that FA profiles were distinct between species when compared under the same cut/management. However this distinction became less clear when comparing species across cuts, which may be a result of the highly significant genotype x cutting date interaction ($P<0.001$), which was also found in the present study. These results additionally demonstrate that differences between total and individual FAs can also exist at a genotypic level, which was also recently demonstrated by Hegarty *et al* (2013). However it is important to highlight the highly significant genotype x cut interactions found here and in the other studies. Increasing knowledge and understanding of how genetics and environment interact with each other is an important step in creating grass varieties with increased FA content.

5.5.3 Chlorophyll vs. Fatty Acids

As detailed earlier, the *in vivo* and *in vitro* chlorophyll results correlated poorly. This in turn resulted in poor correlations being found between *in vivo* chlorophyll and FA content. To the author's knowledge, no other attempts have been made to link *in vivo* chlorophyll measures with FA content; but attempts have been made to link *in vivo* chlorophyll to other nutritional characteristics of forage. Gáborčík (2003) investigated the relationship between *in vivo* chlorophyll and N content of five temperate grass species. They reported R^2 value of 0.49 to 0.94 for individual cuts and an overall R^2 value of 0.84 ($P<0.01$). A more recent study by Hughes *et al* (2014), involving the tropical forage *Bracharia decumbens*, also investigated the relationship

between *in vivo* chlorophyll (measured via the non-destructive handheld chlorophyll gun FieldScout CM 1000 NDVI meter) with IVOMD, lignin and ADF in addition to CP. They reported R^2 values ranging from 0.49 to 0.90 for CP, 0.25 to 0.72 for ADF, 0.16 to 0.66 for lignin and 0.50 to 0.78 for IVOMD.

A small number of studies have investigated the relationship between *in vitro* chlorophyll and FA content in temperate forages. Mayland *et al* (1976) reported positive correlation coefficients for chlorophyll vs. TFA content of 0.89, 0.93, 1.00 and 0.96 for 1st cut perennial ryegrass, 2nd cut perennial ryegrass, wheatgrass and wheat, respectively. Similar results were also reported by Dierking *et al* (2010) for orchard grass (r = 0.89), tall fescue (r = 0.88), perennial ryegrass (r = 0.93) and alfalfa (r = 0.80). Dierking *et al* (2010) also presented the relationship between chlorophyll and C18:3n-3 content which heralded very similar correlation coefficients to that of chlorophyll vs. TFA content. Results of the present study are in line with these, where the correlation coefficients for chlorophyll vs. TFA and C18:3n-3 across all cuts were 0.86 and 0.87, respectively. The present study also revealed high correlations for chlorophyll vs. C16:0 and C16:1*trans*-3, moderate correlations for chlorophyll vs. C18:0 and C18:2n-6 and a low correlation between chlorophyll and C18:1*cis*-9. In terms of correlations between chlorophyll and FAs at each harvest, chlorophyll vs. TFA, C18:3n-3, C16:1*trans*-3 and C16:0 were consistently high across the season. These high and consistent correlations between *in vitro* chlorophyll and FA content suggests that *in vitro* chlorophyll could be used as a reasonably reliable indicator of FA content across the growing season. However, further investigation and validation is needed to develop reliable methods of accurately estimating chlorophyll content of narrow-leaved plants *in vivo*, and in turn estimating FA content.

5.6 Conclusions

The results presented in this chapter shows that total and individual FA content generally increases in leaf tissue during a growing season. Total chlorophyll content was also found to generally increase during the growing season, owing to the positive relationship that exists between FAs and chlorophyll content of plants. Although *in vitro* chlorophyll content correlated well and consistently with TFA and some individual FAs across cuts, the correlations found between *in vivo* chlorophyll (*SPAD*) and *in vitro* chlorophyll were rather poor. Further work is needed to address the issues surrounding the use of chlorophyll meters (e.g. *SPAD*-502) with narrow-leaved plants such as perennial ryegrass, especially concerning validation of the relationship between *SPAD* and *in vitro* chlorophyll content in these plants. This work also demonstrates that differences in FA content and composition exist at a genotypic level, and these differences are somewhat consistent, with low FA genotypes generally staying low and vice versa for high FA genotypes across the growing season. This further establishes the genetic influence on FA content and the potential to selectively breed for this trait.

Chapter 6. Relationships between Individual and Total Fatty Acid Content and Composition

6.1 Summary

The FA data generated from the field study (Chapter 5) was used to establish the relationships between content and proportions of individual and total FAs. In terms of FA content, the majority of FAs positively correlated with each other across the growing season, however some correlations were much more consistent between cuts than others. With regard to FA proportions, an interesting pattern was observed with most FAs correlating positively, apart from with C16:1*trans*-3 and C18:3n-3 which correlated negatively with all other FAs apart from each other. Some of these relationships can be explained by the FA and lipid biosynthesis pathways of plants while other remain unresolved. Further investigation is required to verify these results and establish the control points of FA content and composition, and how genetic and environmental factors may beneficially be exploited to alter the FA content and composition of perennial ryegrass.

6.2 Introduction

Although many authors have investigated the temporal changes in FA content and composition of forages (Dewhurst *et al.*, 2001; Boufaïed *et al.*, 2003; Elgersma *et al.*, 2003a; Palladino *et al.*, 2009); none have gone as far as to investigate the relationships between individual and total FAs or how these vary across a growing season. Establishing what relationships exist between FAs and gaining better understanding of these inter-relationships may give some insight into the regulation of FA and lipid synthesis in grasses. Additionally, the opportunity to examine these relationships

across multiple harvests may also provide insight into how the dynamics of FA and lipid synthesis change across a growing season. Thus, the aim of this chapter was to determine the relationships that exist between the content and proportions of individual and total FAs in perennial ryegrass across a growing season. These inter-relationships would be coupled with current knowledge of FA and lipid synthesis in plants with the intention of explaining the biological basis of some of these relationships.

6.3 Materials and Methods

6.3.1 Fatty Acid Data

The FA data generated from the experiment described in Chapter 5 was used to determine the relationships that exist between the concentrations and proportions of individual and total FAs. The experiment consisted of four Aurora x AberMagic F1 mapping population genotypes and twenty B674G intermediate heading 13th generation breeding population genotypes, with four replicates of each genotype giving a total of ninety-six plants. These were subjected to a simulated grazing management regime which began in May 2013. A total of five harvests were collected and analysed for FA content which took place on 5th June (Cut 1), 1st July (Cut 2), 1st August (Cut 3), 28th August (Cut 4) and 24th September (Cut 5). Extraction and methylation of FAs was carried out on freeze-dried material following the procedure of Sukhija and Palmquist (1988) and quantified via GC-FID (see Chapter 3 for details).

6.3.2 Statistical Analysis

Individual plant data was used to quantify the relationships between content of FAs and proportions of FAs. Data points that were more than 3 standard deviations away from the mean were deemed outliers and omitted from the FA dataset. Spearman's

rank correlation and scatter plot matrices were generated using R software (). Correlations were calculated between C12:0, C14:0, C16:0, C16:1*trans*-3, C18:0, C18:1*cis*-9, C18:2n-6, C18:3n-3 and TFA content and proportions.

6.4 Results

6.4.1 Fatty Acid Content

Spearman's rank correlation and scatter plot matrices for Cut 1, 2, 3, 4 and 5 are presented in Figure 6.1 through to Figure 6.5, respectively. Some FA correlations varied considerably between cuts while others remained fairly consistent across all five cuts. Lauric acid (C12:0) and C14:0 had moderate to high, positive correlations for all five cuts, with values ranging from 0.59 to 0.78 ($P<0.001$). However, correlations between C12:0 and all other FAs were much more variable between cuts, with many correlations being negligible. In general, negative correlations were found between C12:0 and C16:0, C16:1*trans*-3, C18:1*cis*-9, C18:3n-3 and TFA while correlations for C12:0 vs. C18:0 and C12:0 vs. C18:2n-6 were mostly positive. Similar results were also found for correlations between C14:0 and all other FAs, with the majority of correlations being negligible but variable between cuts. However, correlations for C14:0 vs. C16:1*trans*-3 and C14:0 vs. C18:3n-3 were consistently insignificant across all five cuts ($P>0.05$).

Palmitic acid (C16:0) correlated positively with all other FAs apart from C12:0 and C14:0. Correlations were significant across all five cuts between C16:0 and C16:1*trans*-3 ($P<0.001$), C18:0 ($P<0.05$), C18:3n-3 ($P<0.001$) and TFA ($P<0.001$), ranging from 0.51 to 0.83, 0.21 to 0.58, 0.82 to 0.92 and 0.90 to 0.96, respectively. Moderate correlations were also observed for C16:0 vs. C18:1*cis*-9 ($P<0.05$) and C16:0 vs. C18:2n-6 ($P<0.001$), with the exception of Cut 3. Variable correlations were

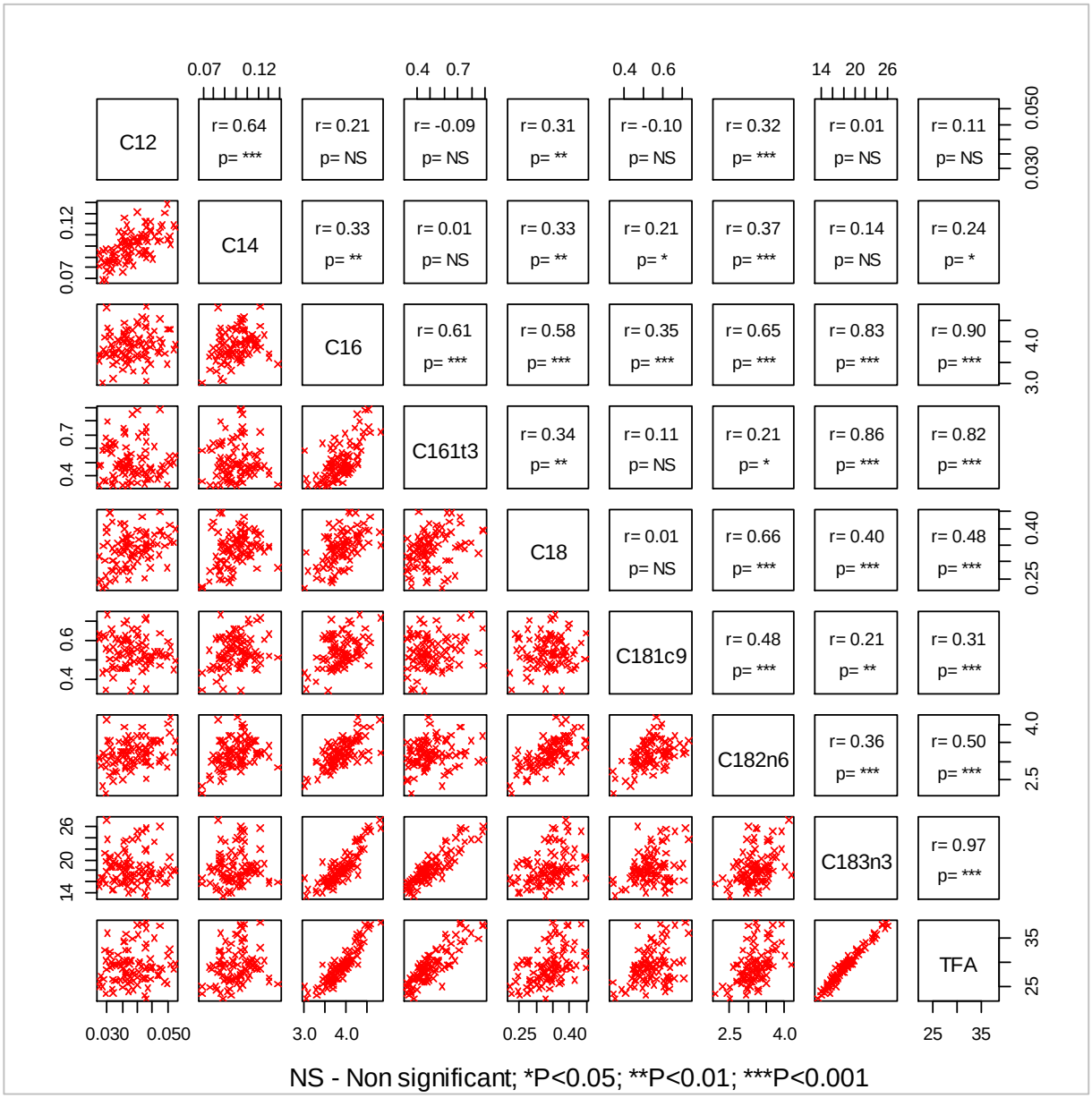

Figure 6.1 Scatter plot and spearman's rank correlation matrix showing the relationships between content of individual and total fatty acids (TFA) at Cut 1

Figure 6.2 Scatter plot and spearman's rank correlation matrix showing the relationships between content of individual and total fatty acids (TFA) at Cut 2

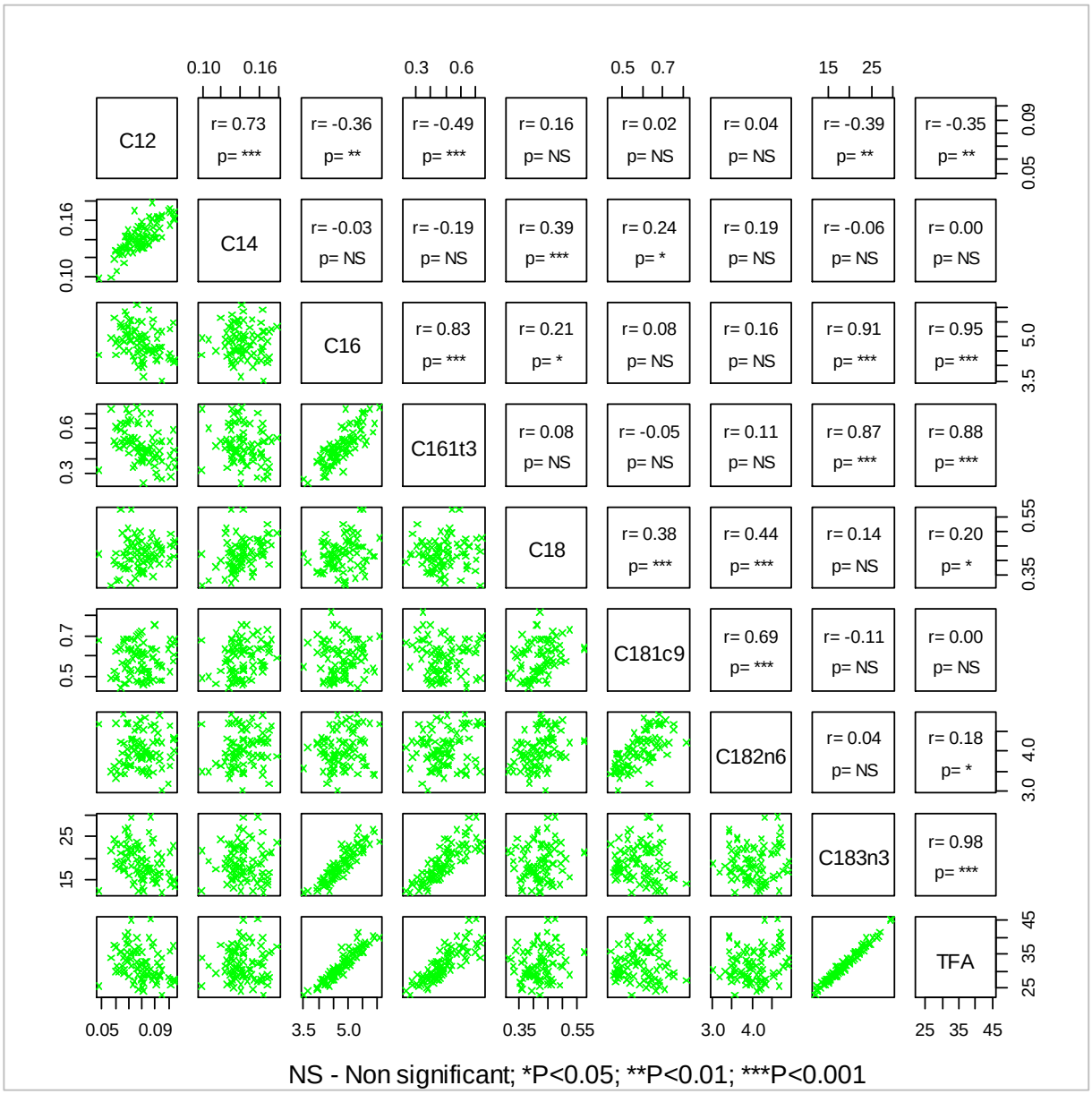

Figure 6.3 Scatter plot and spearman's rank correlation matrix showing the relationships between content of individual and total fatty acids (TFA) at Cut 3

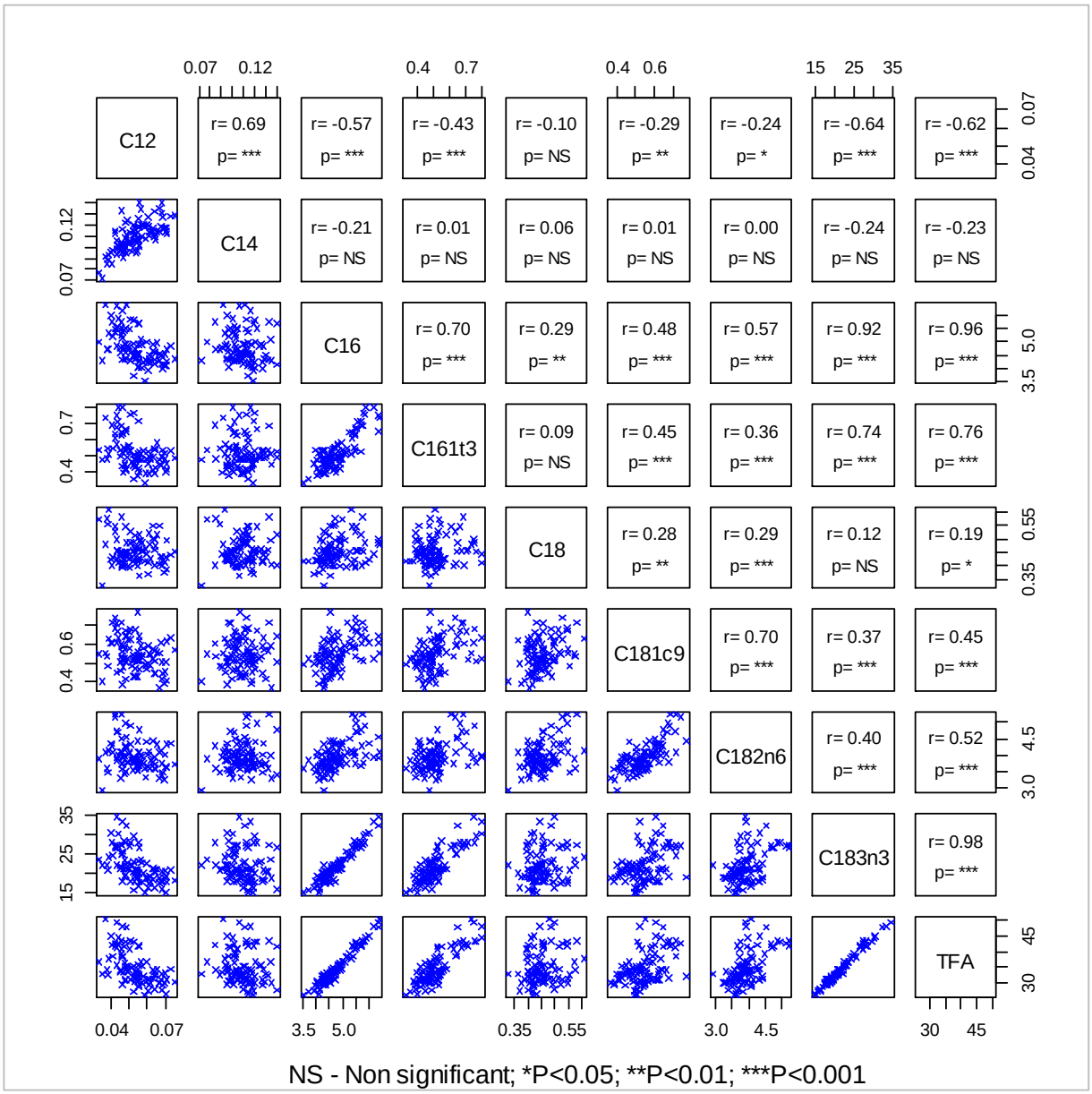

Figure 6.4 Scatter plot and spearman's rank correlation matrix showing the relationships between content of individual and total fatty acids (TFA) at Cut 4

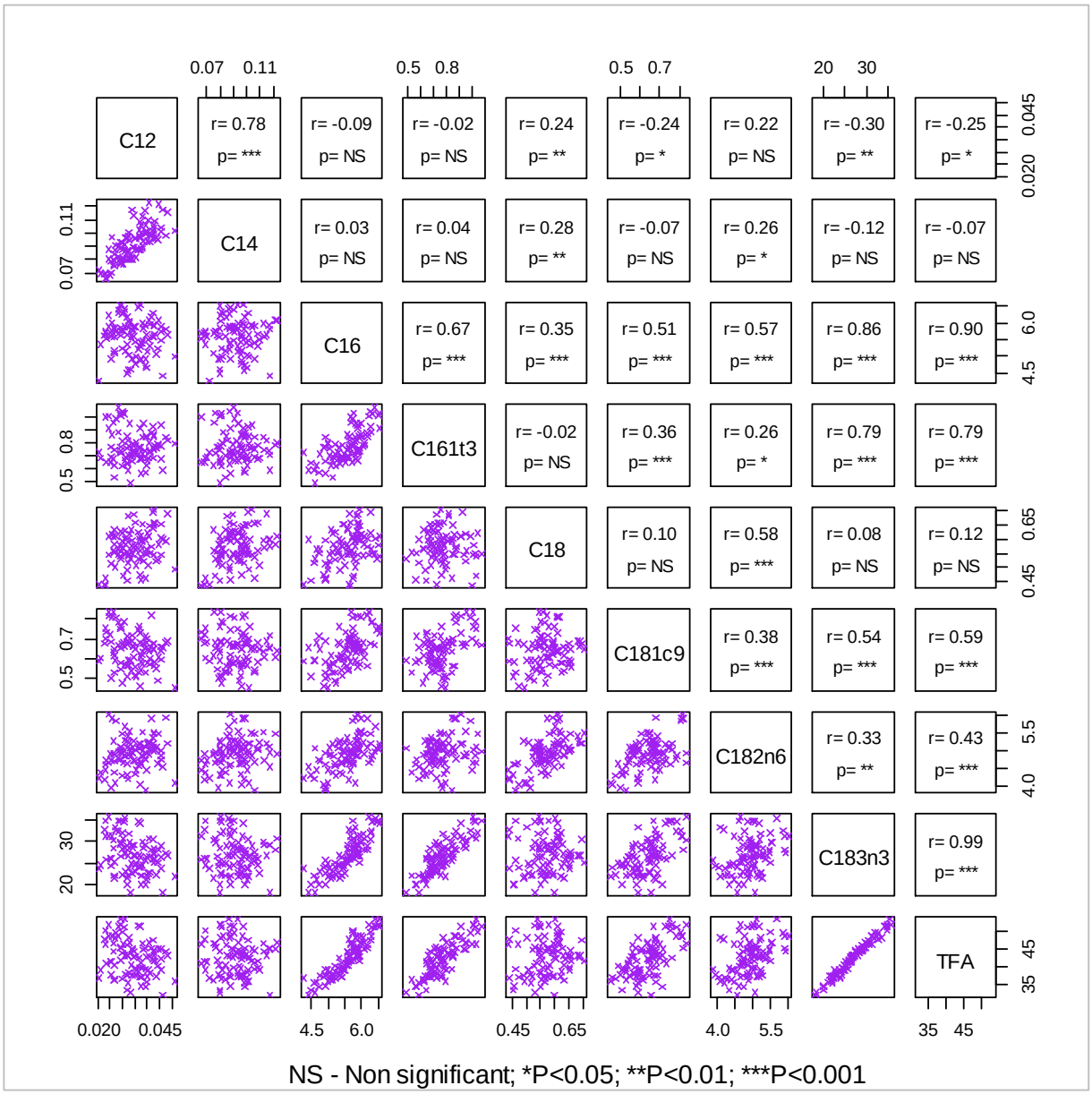

Figure 6.5 Scatter plot and spearman's rank correlation matrix showing the relationships between content of individual and total fatty acids (TFA) at Cut 5

found between C16:1*trans*-3 and C18:0, C18:1*cis*-9 and C18:2n-6, with many of these being insignificant. However, highly positive correlations were found for C16:1*trans*-3 vs. C18:3n-3 (r=0.73 to 0.87; P<0.001) and C16:1*trans*-3 vs. TFA (r=0.70 to 0.88; P<0.001).

Stearic acid (C18:0) correlations also fluctuated between cuts, apart from the moderate correlation between C18:0 and C18:2n-6 which ranged from 0.29 to 0.66 (P<0.001) and remained fairly consistent across the five cuts. Interestingly, the correlation between C18:0 and TFA decreased steadily from 0.48 at Cut 1 (P<0.001) to 0.12 at Cut 5 (P>0.05). Oleic acid (C18:1*cis*-9) also correlated consistently with C18:2n-6 across all five cuts, ranging from 0.38 to 0.70 (P<0.001). The correlations of C18:1*cis*-9 with C18:3n-3 and TFA varied between cuts but were especially low at Cut 3 (P>0.05). Moderate correlations were found between C18:2n-6 and C18:3n-3 (P<0.01), except for Cut 2 and 3. There was also a weakening in the correlation between C18:2n-6 and TFA at Cut 2 and 3, however the correlations between these FAs remained significant across all cuts (P<0.05). Exceptionally strong correlations were found between C18:3n-3 and TFA, which remained extremely consistent between cuts and ranged between 0.97 and 0.99 (P<0.001).

6.4.2 Fatty Acid Proportions

The correlation matrices presented in Figure 6.6 through to Figure 6.10 show the relationships between the proportions of individual FAs for Cuts 1, 2, 3, 4 and 5, respectively. In general, the majority of FA proportions were positively correlated; with the exceptions of C16:1*trans*-3 and C18:3n-3. Conversely, these FAs were negatively correlated with the proportions of all other FAs, apart from each other. Although not all correlations were significant, this pattern was observed across all five cuts.

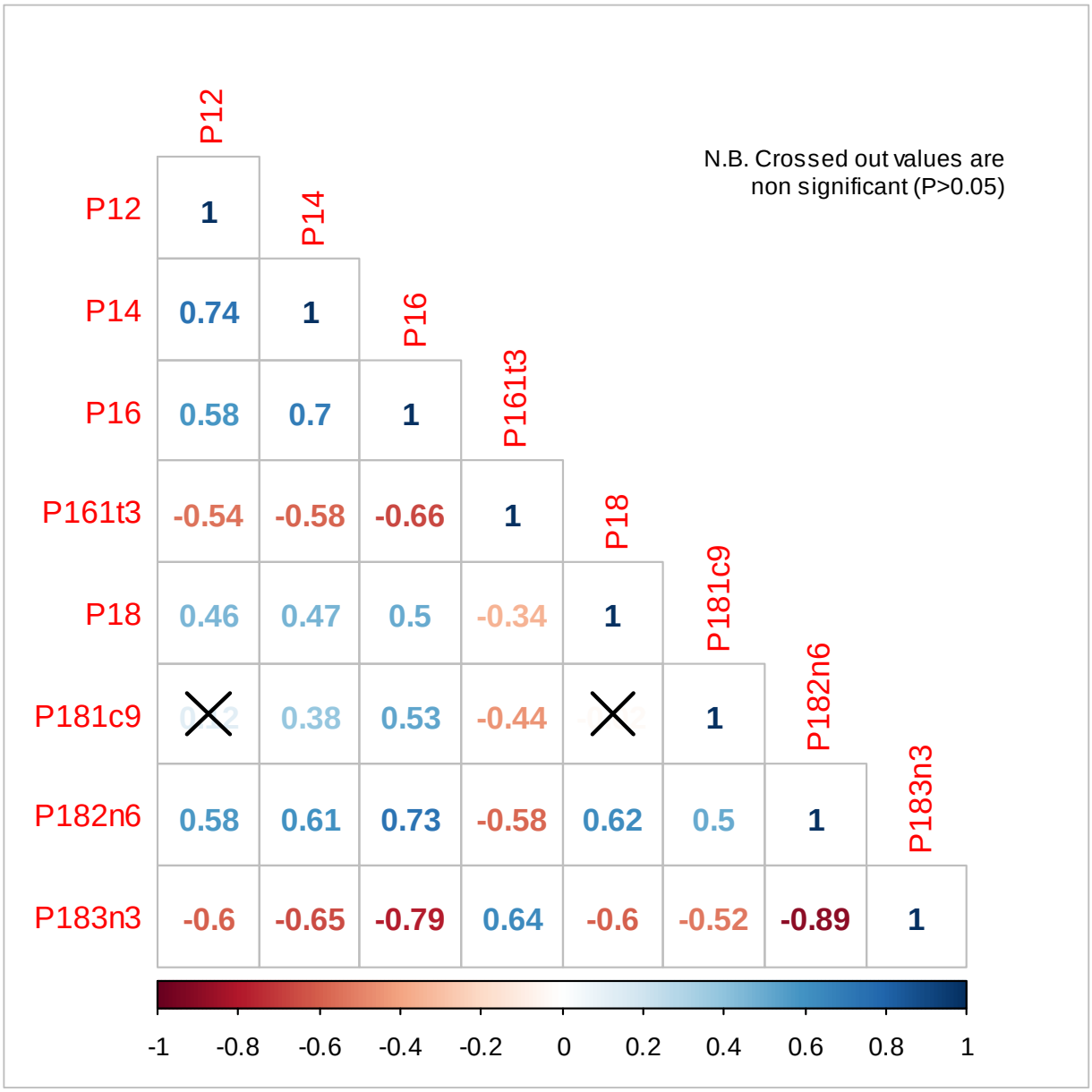

Figure 6.6 Spearman's rank correlation matrix of the relationships between the proportions of individual and fatty acids at Cut 1

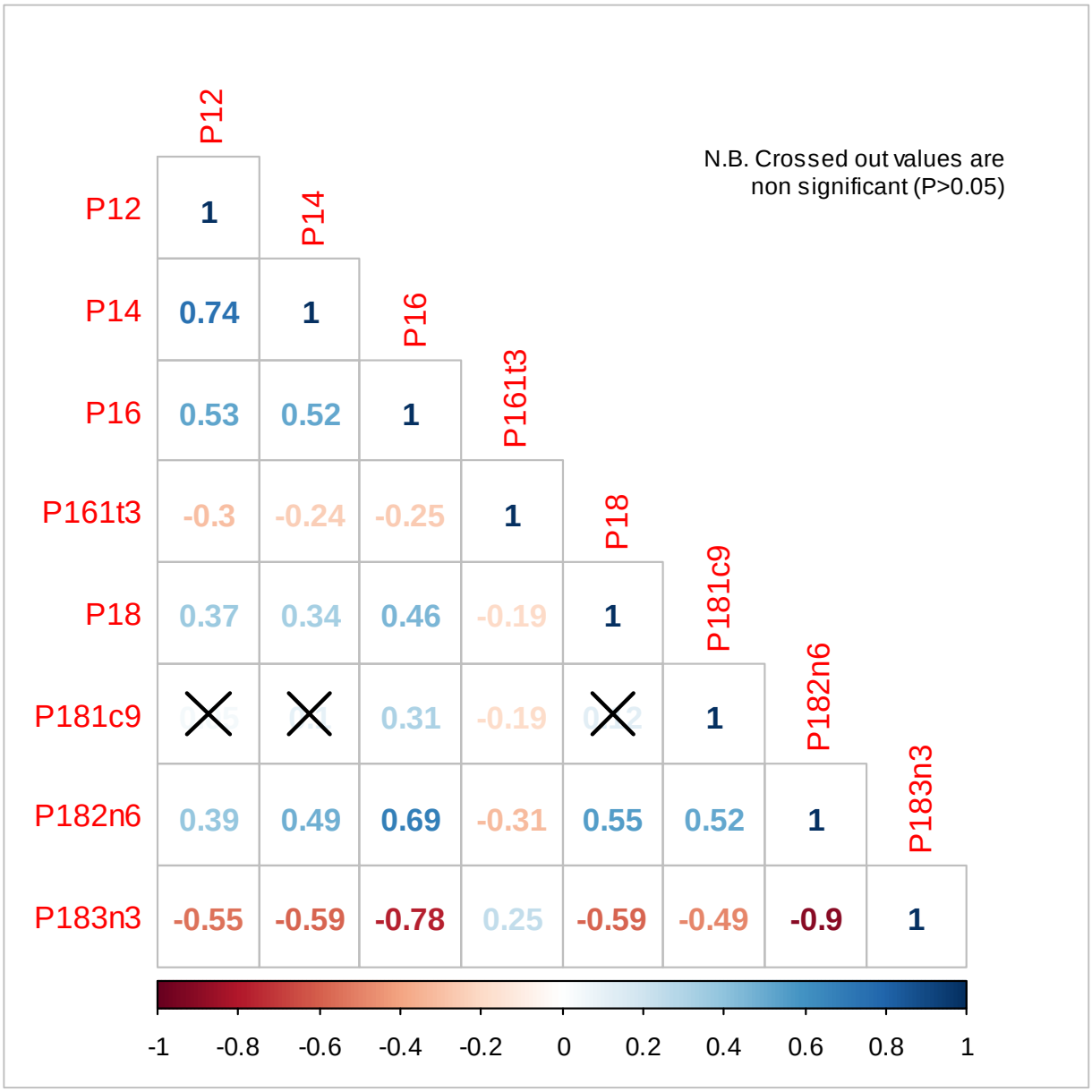

Figure 6.7 Spearman's rank correlation matrix of the relationships between the proportions of individual fatty acids at Cut 2

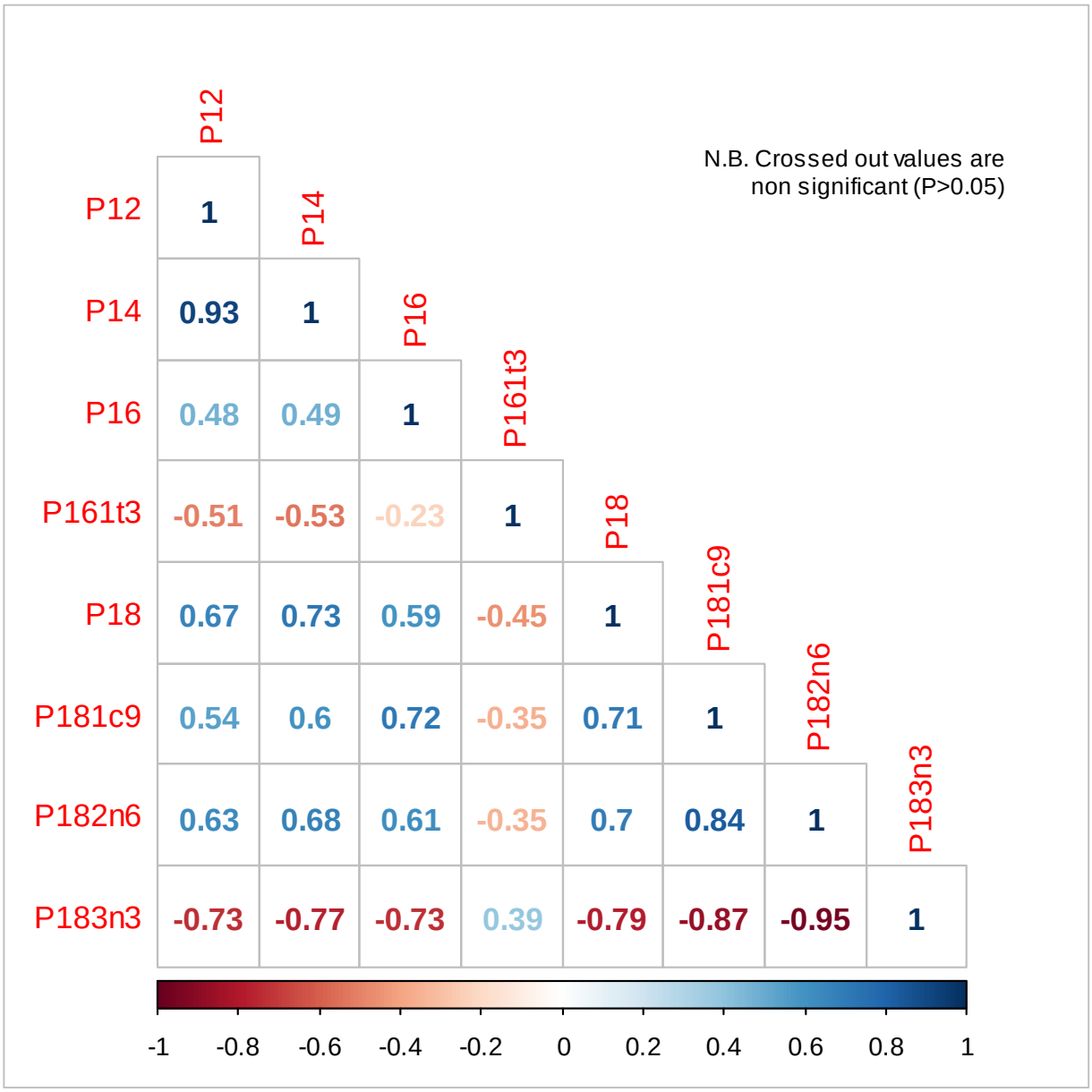

Figure 6.8 Spearman's rank correlation matrix of the relationships between the proportions of individual fatty acids at Cut 3

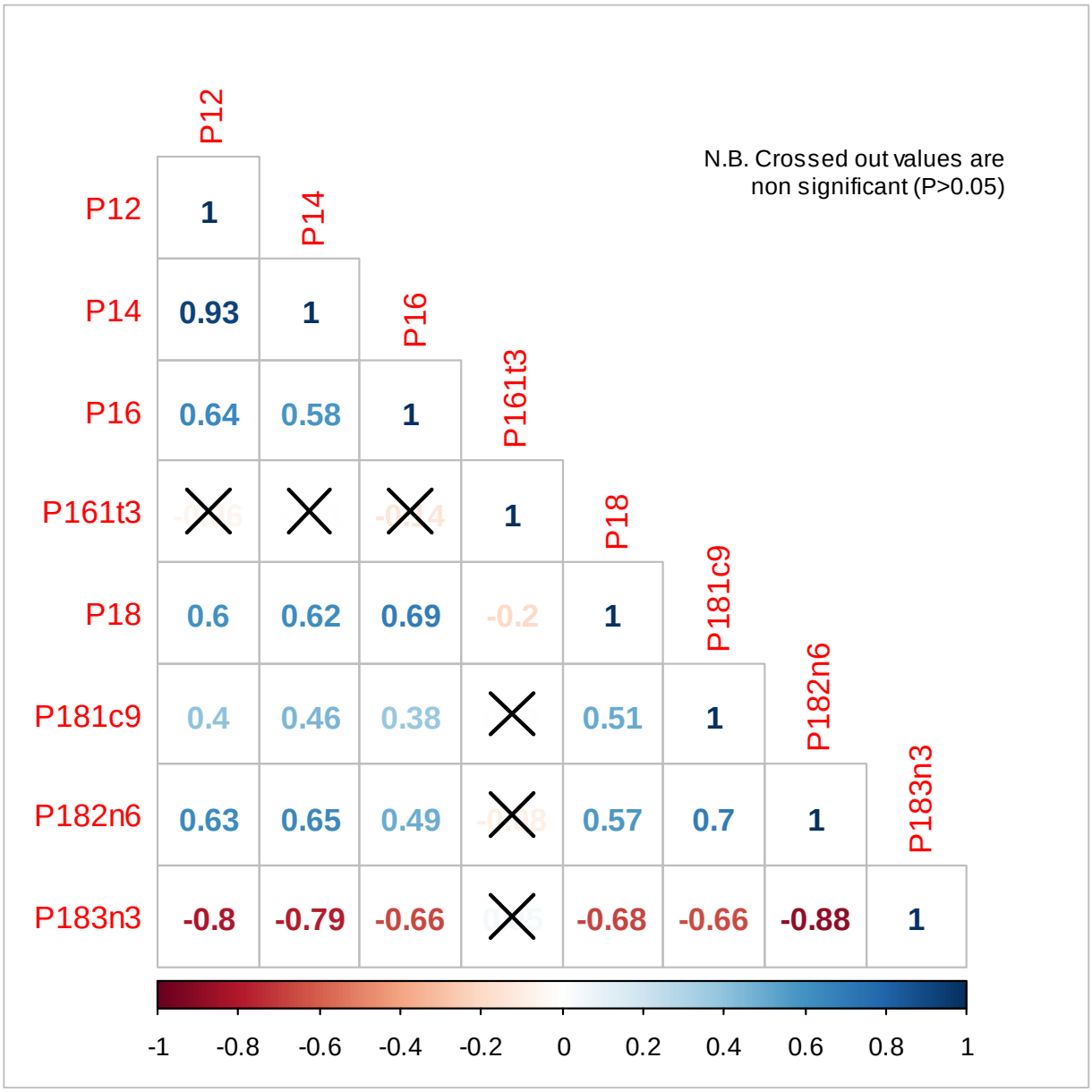

Figure 6.9 Spearman's rank correlation matrix of the relationships between the proportions of individual fatty acids at Cut 4

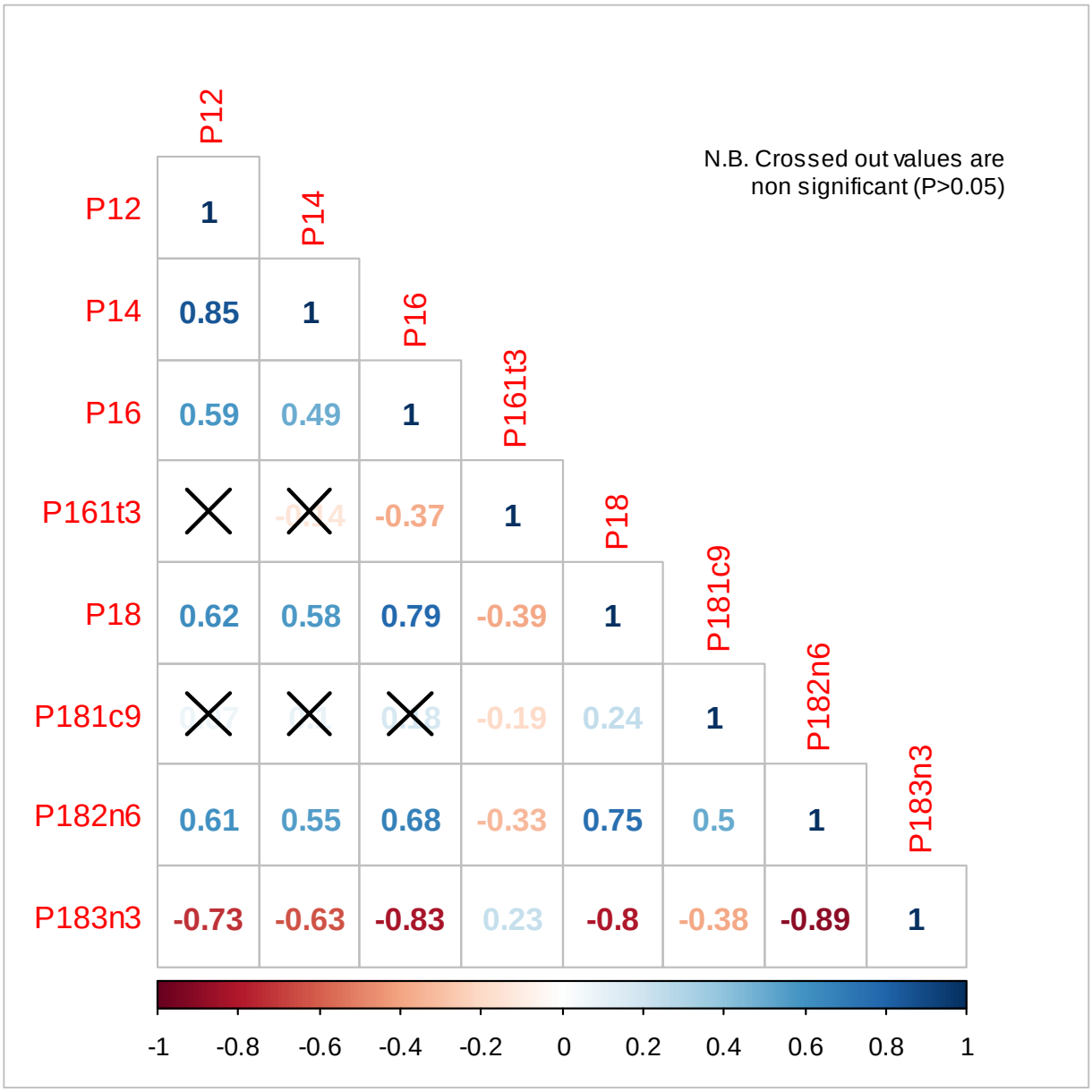

Figure 6.10 Spearman's rank correlation matrix of the relationships between the proportions of individual fatty acids at Cut 5

Many of the correlations between FA proportions remained consistently high across all five cuts. Positive correlations were found for C12:0 proportion (P12:0) vs. C14:0 proportion (P14:0), C16:0 proportion (P16:0) and C18:2n-6 proportion (P18:2n-6), which ranged from 0.74 to 0.93, 0.48 to 0.64 and 0.39 to 0.63 across all five cuts ($P<0.001$). A strong positive correlation was also found across all cuts for P12:0 vs. C18:0 proportion (P18:0), ranging from 0.37 to 0.67 ($P<0.01$). All correlations between P14:0, P16:0, P18:0 and P18:2n-6 were positive with moderate to high values; with the lowest r value being 0.34 for P14:0 vs. P16:0 at Cut 2 and the highest r value of 0.79 for P16:0 vs. P18:0 at Cut 5. However, C18:3n-3 proportion (P18:3n-3) correlated negatively with P12:0, P14:0 and P16:0, ranging from -0.55 to -0.80, -0.59 to -0.79 and -0.66 to -0.83, respectively. Correlations relating to C16:1*trans*-3 proportion (P16:1*trans*-3) were on the whole low and considerably variable between cuts, yet all were negative apart from with P18:3n-3. Correlations between C18:1*cis*-9 proportion (P18:1*cis*-9) and P12:0, P14:0, P16:0 and P18:0 were positive but also varied between cuts. Proportion of C18:0 (P18:0) and P18:1*cis*-9 both had consistently positive correlations with P18:2n-6, ranging from 0.55 to 0.75 and 0.50 to 0.84, respectively. Whereas P18:3n-3 correlated negatively with these FAs, ranging from -0.59 to -0.80 for P18:0 vs. P18:3n-3 and -0.38 to -0.87 for P18:1*cis*-9 vs. P18:3n-3 ($P<0.001$). Very high negative correlations were found between P18:2n-6 and P18:3n-3 of -0.88 to -0.95 ($P<0.001$).

6.5 Discussion

6.5.1 Fatty Acid Content

The positive correlations between content of FAs (excluding some C12:0 and C14:0 correlations), suggests that there is generally a parallel increase in the synthesis of all individual FAs, resulting in an increase in TFA content. This agrees with the belief that one enzyme (ACCase) exerts the main flux control over FA synthesis in plants (Harwood, 1996; Ohlrogge and Jaworski, 1997). Nonetheless, some correlations varied considerably between cuts while other remained consistent across all cuts. This demonstrates that environmental factors can greatly influence the relationships between certain individual FAs while others are seemingly unaffected.

The correlations between C12:0 and C14:0 were stable across cuts, which would be expected as C12:0 is converted to C14:0 within the plastid via KASI. No further consistent correlations were found for C12:0 or C14:0 with any of the other reported FAs. Surprisingly, the correlations between C14:0 and C16:0 were low across all cuts despite these two FAs being biosynthetically related via the KASI enzyme as well. This difference in strength of relationship between C12:0 vs. C14:0 and C14:0 vs. C16:0 may be due to a difference in substrate affinity by the KASI enzyme. Alternatively, one of the other KAS isoenzymes may also be catalysing this condensation reaction. Harwood (2005) states that, although the chief action of KASIII is producing 4C acyl-ACP, it can probably catalyse further condensation reactions, while Buchanan *et al* (2007) contrastingly state that KASII is able to accept 10-16C acyl-ACPs; therefore which of the two KAS isoenzymes is possibly catalysing this conversion alongside KASI is uncertain. The conversion of C16:0 to C18:0 is catalysed by KASII in plants. These two FAs had moderate correlations with each

other, however a slight weakening of this relationship was observed at Cut 3 and Cut 4. Reasons for this are unclear but may be due to environmental factors such as the relatively dry month leading up to Cut 3 harvest.

High correlations were found across all cuts between C16:0 and C16:1*trans*-3. The fatty acid desaturase (FAD) responsible for introducing the *trans*-3 double bond into C16:0 is FAD4, which acts specifically on plastid PG (Gao *et al.*, 2009). This implies that perennial ryegrass has the ability to synthesis PG via the 'prokaryotic' lipid synthesis pathway, which takes place within the plastid. However, the absence of C16:3n-3 shows that it cannot produce any further lipids via this pathway, and accordingly perennial ryegrass is classified as a 'C18:3n-3' plant. The key difference between 'C16:3n-3' plants, where galactolipids are produced via both 'prokaryotic' and 'eukaryotic pathways, and 'C18:3n-3' plants, where galactolipids are produced only by the 'eukaryotic' pathway, is the presence or absence of phosphatidate phosphatase (PAP) activity (Mongrand *et al.*, 1998).

Palmitic acid (C16:0) correlated well with C18:1*cis*-9, C18:2n-2, C18:3n-3 and TFA, despite the fact that it is not immediately related to these FAs from a FA biosynthesis point of view. These relationships are probably a result of C16:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3 being the major FAs occupying lipid synthesis in the ER and also contribute to the majority of TFA.

Interestingly, high correlations were found in the present study between C16:1*trans*-3 with C18:3n-3 and TFA. Referring back to the lipid synthesis schematic from Chapter 2 (Figure 2.5), C16:1*trans*-3 is specifically esterified to the *sn*-2 position of the PG glycerol backbone (Gao *et al.*, 2009). Whereas 18C FAs are present at the *sn*-1 position, of which, C18:3n-3 is the ultimate 18C FA. Thus, a strong and consistent

relationship is formed between C16:1*trans*-3 and C18:3n-3 due to the co-esterification of these FAs to PG.

Considering that C18:0 and C18:1*cis*-9 are closely associated in terms of FA synthesis via the stearoyl-ACP desaturase enzyme, correlations between these FAs were negligible, apart from at Cut 3 and Cut 4. However C18:0 correlated moderately with C18:2n-6 across all five cuts. Also, an interesting pattern was observed between correlations of C18:0 with C18:3n-3 and TFA where the strength of these relationships decreased from Cut 1 through to Cut 5. Moderate to high correlations were observed between C18:1*cis*-9 and C18:2n-6 across all cuts since C18:1*cis*-9 is converted to C18:2n-6 via FAD2. Oleic acid (C18:1*cis*-9) and C18:2n-6 did not have any consistent correlations with C18:3n-3 and TFA across cuts but there were noticeable decreases in these relationships at Cut 2 and Cut 3 in particular. Reasons for these variable correlations are uncertain. Exceptionally high and very consistent correlations were found between C18:3n-3 and TFA across all cuts. This is predominantly a result of such a high proportion of TFA being in the form of C18:3n-3 (Hawke, 1973).

6.5.2 Fatty Acid Proportions

In contrast to the FA content relationships, the relationships between proportions of individual FAs were unexpectedly consistent. Although not all correlations where significant, the same pattern was found across all five cuts. It appears that the proportions of C16:1*trans*-3 and C18:3n-3 are the chief regulators over the proportions of the other FAs, from the fact that both FAs negatively correlated with all other FAs, apart from each other; whereas all other FA proportions were positively correlated. This may imply that regulation of FA composition is important in plants. Indeed, FAs are the major constituents of lipids; the vast majority of which form the basic

components of cellular membranes. Accordingly, the composition of FAs present in these lipids affects the structure, fluidity and functionality of the lipid membranes.

The most common example of the importance of FA composition with regards to optimal plant growth and development is temperature sensitivity of plants (Taiz and Zeiger, 2007). Generally, a decrease in growth temperature causes increased unsaturation of FAs (Nishida and Murata, 1996). Changes in FA composition particularly take place in the thylakoid membranes, as the PUFA within these membranes play an important structural feature in photosynthesis and an essential role in the maintenance of the electron transport system (Williams *et al.*, 1983; Routaboul *et al.*, 2000). Interestingly however, in contrast to cyanobacteria these changes in FA composition in response to temperature changes are not transcriptome driven (in *Arabidopsis* at least, with the exception of *fad8*) suggesting that this change is controlled by several different desaturase steps within the FA and lipid biosynthesis pathways (Falcone *et al.*, 2004; Buchanan *et al.*, 2007).

6.6 Conclusions

Concerning the results presented here, it would seem that the desaturases controlling the conversion of C18:2n-6 to C18:3n-3 and to a lesser extent C16:0 to C16:1*trans*-3 also exert some control on the overall FA composition under temperate conditions. Some interesting relationships and dynamics between the content and proportions of individual and total FAs have been uncovered. However, further investigation is required to verify these results and establish the control points of FA content and composition, and how genetic and environmental factors may beneficially be exploited to alter the FA content and composition of perennial ryegrass.

Chapter 7. Development of a Lipid Analysis Method to Investigate Relationships between Chlorophyll, Fatty Acids and Lipids

7.1 Summary

Plant material from the first harvest of the field study discussed in Chapter 5 was used to investigate whether genotypes with elevated TFA content and chlorophyll content had increased proportions of galactolipids. This required the set-up of a new laboratory method to facilitate investigation of the lipid composition of the samples; the development of which forms the main body of this chapter. Nonetheless, Strong, positive correlations were found between TFA, chlorophyll and proportion of galactolipids ($r \geq 0.69$; $P<0.001$); whereas phospholipid proportion and neutral lipid proportion were negatively correlated with TFA, chlorophyll and galactolipids. This work reveals that genotypes with increased TFA content have increased proportions of galactolipid, in addition to increased chlorophyll content. However, a great deal more research is needed into the lipid composition of perennial ryegrass, along with other forages, to (a) validate the results presented here, (b) establish the mechanisms behind the increased galactolipids, (c) investigate environmental effects on lipid composition and metabolism, and (d) establish an understanding of the genetic control underpinning lipid composition of forages.

7.2 Introduction

Plant lipids can be broadly separated into three main groups, namely the neutral lipids, phospholipids and galactolipids. Phospholipids are generally associated with non-photosynthetic tissues such as roots and usually contain high proportions of C16:0

and C18:1*cis*-9 (Murata *et al.*, 1982; Yoshida *et al.*, 2007; Hildebrand, 2012). Galactolipids, on the other hand, contain high proportions of PUFA and are most abundant in photosynthetically active leaves. Chloroplast membranes account for 70% of the total membrane lipids in photosynthetic tissues (Taiz and Zeiger, 2007); of which galactolipids form the major lipid components, particularly the thylakoid membranes of the chloroplast lamellae, which are 50% lipid by weight (Hawke, 1973).

Consequently, a positive correlation exists between FAs and chlorophyll, due to their co-localisation within chloroplasts (Hawke, 1973). Correlation coefficients of above 0.8 have been reported by Mayland *et al* (1976) and Dierking *et al* (2010) for chlorophyll vs. TFA content in a variety of different forage species. This relationship has been shown in the work discussed in Chapter 5, which resulted in an across cut average correlation coefficient of 0.86, along with high correlations between chlorophyll and C18:3n-3, C16:0 and C16:1*trans*-3.

In view of the association between chlorophyll and TFA, along with the evidence of genotypic differences in TFA content presented in Chapter 5, this poses the question of whether the higher TFA genotypes have an increased amount of thylakoid membranes per chloroplast or an increased number of chloroplasts. Indeed, Dierking *et al* (2010) also concluded their study by questioning whether an increase in either (a) the amount of thylakoid membranes contained within chloroplasts or (b) the number of chloroplasts themselves would result in an increase in FA content. The aim of the present study was to investigate the relationships between chlorophyll content, FA content and the lipid composition of perennial ryegrass. If an increase in TFA content is due to increased chloroplast membrane, it is hypothesised that the genotypes with higher FA content would have a higher proportion of galactolipids. However, to achieve the necessary separation of lipids to investigate this hypothesis required a new

laboratory method to be set up. An overview of methods used to achieve separation of galactolipids and phospholipids was presented and discussed in Chapter 2.

The current chapter focusses on the method development aspect which involved establishing, testing and adjusting the chosen method with the use of purified lipid standards and non-experimental perennial ryegrass material ('test grass') prior to analysis of the experimental material.

7.3 Method Development

7.3.1 Plant Material

Lyophilised plant material from Cut 1 of the field experiment described in Chapter 5 was used. Bulk composite samples of each genotype were obtained by weighing out equal amounts of each of the four replicates and combining these together, to give a total of 24 samples each representing one genotype.

7.3.2 One-Dimensional Thin-Layer Chromatography (1D-TLC)

The 1D-TLC method published by Nichols (1963) was used to separate total lipid into four fractions: mono- and diacylglycerol (DAG), triacylglycerol (TAG), free fatty acid (FFA) and polar lipid (POL). Approximately 1 g of composite sample was weighed into a culture tube and the exact weight recorded to four decimal places. Five ml of a Chloroform : Methanol ($CHCl_3$:MeOH; 2:1, v/v) solution was added to the tube. Samples were shaken on an orbital shaker at ~300 rpm for 5 min followed by centrifugation at 1500 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube. This process was repeated a further two times giving a total extract volume of approximately 15 ml.

Approximately half of the extract volume (~7.5ml) was transferred into a fresh culture tube and dried under N in a heat block at 50ºC. Meanwhile, 20cm x 20cm 250µm POLYGRAM SIL-G/UV_{254} polyester-backed TLC plates (HiChrom Ltd, Reading, Berkshire, UK) were scored and spotted with standards (DAG, TAG, FFA) on one-third of the TLC plate (see Figure 7.1). Dried extracts were re-dissolved in 1 ml of $CHCl_3$ and pipetted onto the remaining two-thirds of the TLC plate in one continuous fine line (see Figure 7.2). Plates were transferred into a TLC tank containing Acetic acid : Diethyl ether : Hexane (2:30:70) as the mobile phase and left to run for approximately 1 hour (see Figure 7.3). Plates were removed from the TLC tanks and allowed to dry (see Figure 7.4) before spraying with 2,7-dichlorofluorescein and placed in a dark room to develop for ~20 min. Lipids were observed under UV_{254} light and marked with a pencil. Separated lipids were then scraped off into culture tubes and methylated following the Sukhija and Palmquist (1988) method, as described in detail in Chapter 3.

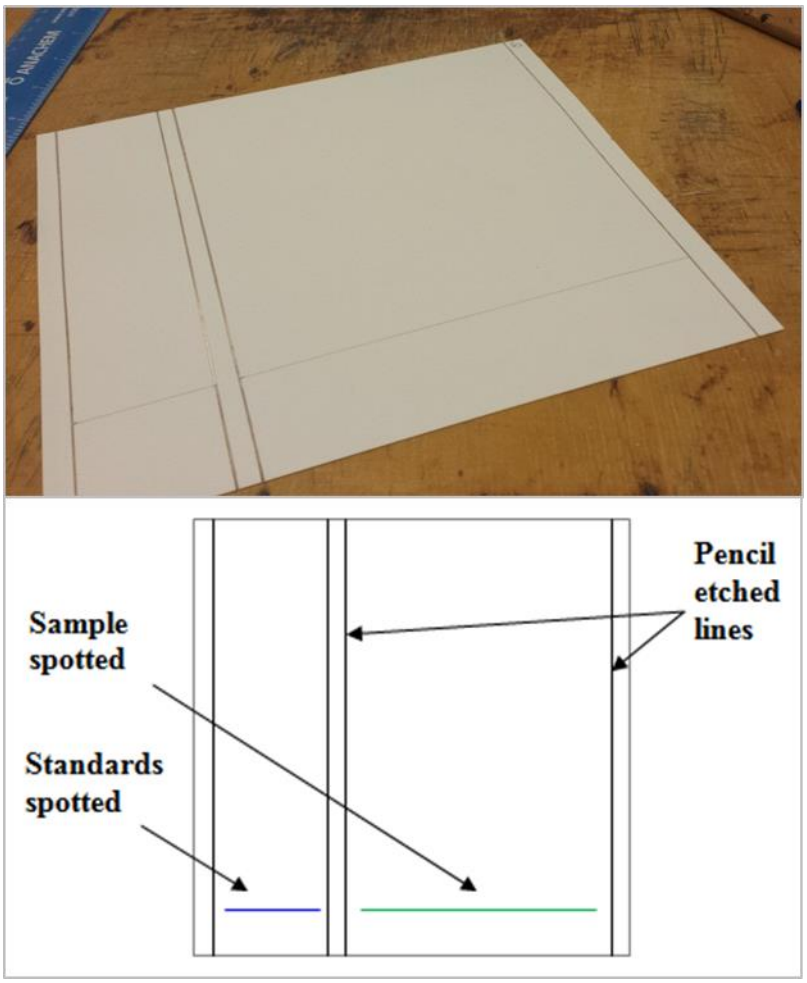

Figure 7.1 Scoring and sample placement on plate for one-dimensional thin-layer chromatography (1D-TLC)

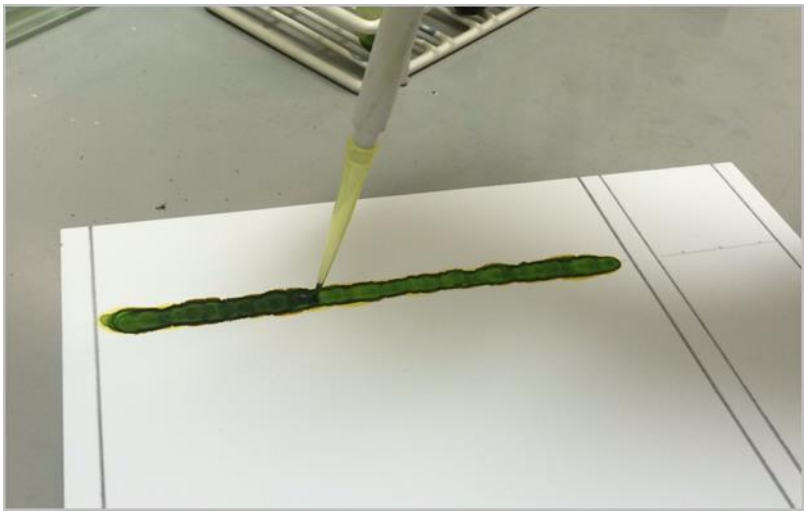

Figure 7.2 Spotting of sample for one-dimensional thin-layer chromatography (1D-TLC)

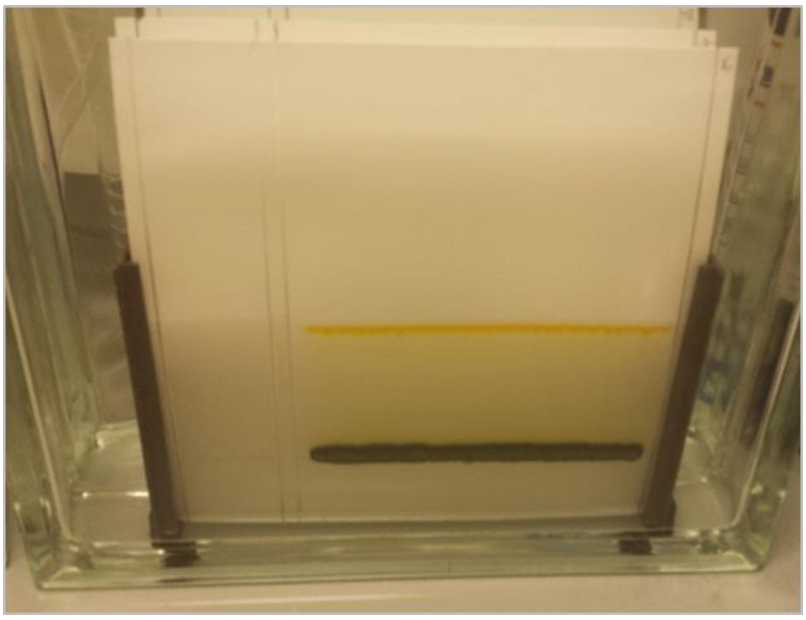

Figure 7.3. Example of a plate mid-way through the 1D-TLC lipid separation

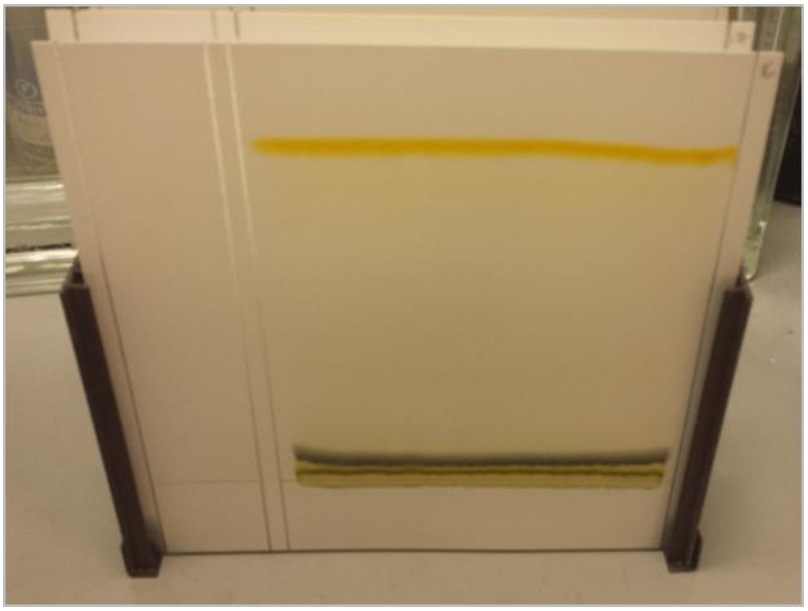

Figure 7.4 Example of a plate after 1D-TLC lipid separation is complete

7.3.3 Two-Dimensional Thin-Layer Chromatography (2D-TLC)

The method outlined by Christie (2003) was selected as the most appropriate and practicable 2D-TLC method for the separation of the phospholipids and galactolipids of grass. This method involves developing the TLC plates using $CHCl_3$: MeOH : H_2O (75:25:2.5, v/v) as the first eluent (Solvent 1). Plates were then dried, rotated 90^o anti-clockwise and developed using a second eluent consisting of $CHCl_3$: MeOH : AA : H_2O (80:9:12:2, v/v) (Solvent 2). Figure 7.5 is the schematic taken from Christie (2003) showing the resulting lipid separation using this technique.

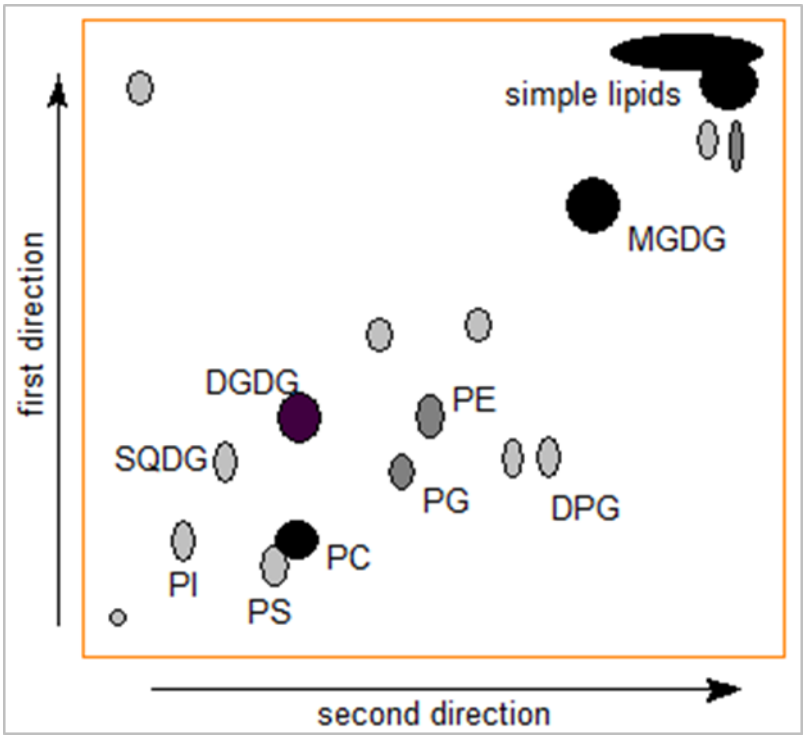

Figure 7.5 Schematic of 2D-TLC separation of grass lipids (from Christie, 2003). Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol (Cardiolipin); PE, phosphatidylethanolamine.

Stage 1. *Test Grass: Familiarising with the Method*

♦ *Test Grass Lipid Extraction and Sample Preparation*

Approximately 0.5 g of freeze-dried perennial ryegrass test sample was weighed out into a culture tube, and exact weight recorded to four decimal places; to which 5 ml of $CHCl_3$: MeOH (2:1) was added. The tube was shaken on an orbital shaker at ~300 rpm for 5 min followed by centrifugation at 1500 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube. This process was repeated a further two times giving a total extract volume of approximately 15 ml. Approximately one-third (~5 ml) of this extract was transferred to a new culture tube using a glass Pasteur pipette and dried down under N at 50^{o}C. The lipid (~5 mg) was then re-suspended in 0.5 ml $CHCl_3$.

♦ *2D-TLC*

An adsorption pad was placed into the TLC development tank, which contained ~1.5 cm depth of Solvent 1, for ~30 min in order to 'prime' the tanks by saturating the atmosphere. In the meantime, the test grass sample was spotted onto a 20cm x 20cm 1mm SIL-G/UV_{254} glass-backed TLC plate (HiChrom Ltd, Reading, Berkshire, UK) ~2.5 cm from the bottom and left-hand edges of the plate (see Figure 7.6), so that the sample would not contaminate the eluent when placed into the TLC development tanks. The TLC plate was then placed onto a rack and into a TLC tank and left to develop. Once the solvent front reached ~2 cm from the top of the plate, the plate was removed from the tank. The run time for Solvent 1 was 3 hr 10 min. After removal of the plate from the TLC tank, it was allowed to air-dry for ~30 min in a fume cupboard and for a further ~30 min in a desiccator to ensure complete evaporation of Solvent 1 prior to the second development of the plate. The plate was then rotated 90^{o} anti-

clockwise and placed into a second TLC development tank containing ~1.5 cm depth of Solvent 2, which had been primed for ~30 min with an adsorption pad. The run time for Solvent 2 was 2 hr 30 min. The plate was removed from the TLC tank, dried in a fume cupboard for ~30 min and then transferred to a desiccator and allowed to dry completely under vacuum overnight.

Figure 7.6 Spotting of the sample for two-dimensional thin-layer chromatography (2D-TLC)

♦ *Visualising and Removing Lipid Fractions*

The plate was removed from the desiccator, sprayed with 2,7-dichlorofluorescin and allowed to develop in a dark room for ~20 min. Lipids were visualised using UV_{254} light and their positions lightly marked with a pencil. Lipids were identified by their relative positions in reference to the schematic shown in Figure 7.5 from Christie (2003). The marked test grass plate with labelled lipids is shown in Figure 7.7. The lipid fractions were then scraped off the plate and placed into individual culture tubes. A spatula was used to break up the silica into a fine powder.

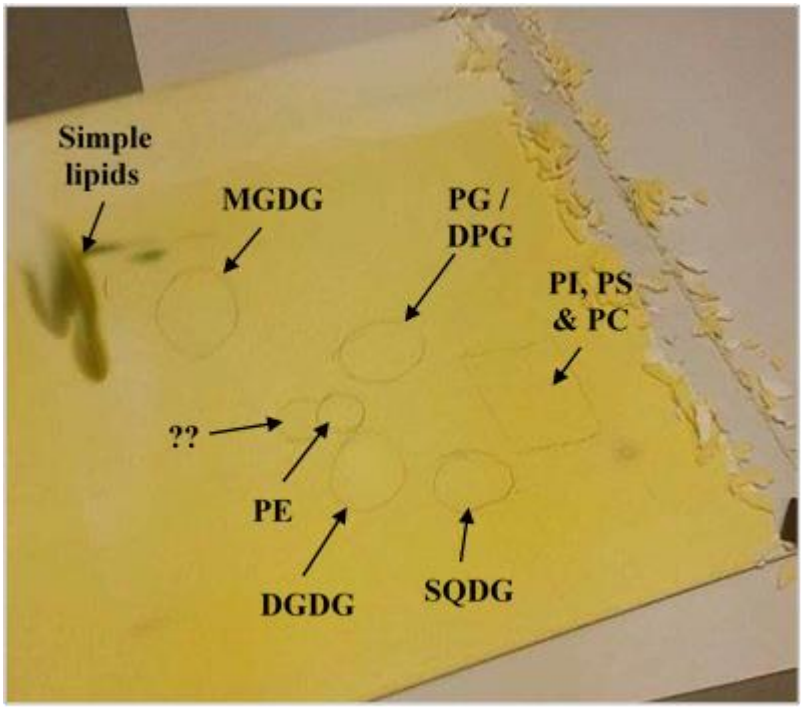

Figure 7.7 First test grass 2D-TLC plate completed run.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol (Cardiolipin); PE, phosphatidylethanolamine; ??, unknown.

♦ *Methylation*

Methylation of the lipid fractions was carried out using the procedure of Sukhija and Palmquist (1988), which is described in detail in Chapter 3. Heneicosanoic acid (C21:0) methyl ester (Sigma-Aldrich Co., USA) in toluene (0.5mg/ml) was used as the internal standard with the customary 2 hr methylation at 70^{o}C. Fatty acids were then quantified using GC-FID following the conditions described in Chapter 3.

♦ *Stage 1 Outcomes*

This first attempt at the 2D-TLC method spanned a total of two-days, due to a total run time of the TLC plates of over 5 hr 30 min plus drying taking up the majority of the first day. As a result, the visualisation, scraping and methylation of the lipid fractions had to be done the following (second) day. Also, some of the lipid spots,

particularly the phospholipids, were not clearly defined when visualised under the UV_{254} lamp. The 'PI, PS and PC' area marked on the plate did not actually show any fluorescence, and thus lipid, when using the UV_{254} lamp. However, according to the schematic in Christie (2003) and relative to the position of the other lipids, the PS, PI and PC fractions should be located in that area. Due to the poor fluorescence and definition of the lipids, it was decided to run a second attempt using the test grass extract but loading a higher amount of lipid onto the plate to try to achieve better fluorescence of the lipids. Alongside this, a plate would be loaded with lipid standards to ensure that the separation and identification of the lipids being achieved was comparable to that shown in the Christie (2003) schematic.

Stage 2. Test Grass and Lipid Standards: Identifying Lipids

♦ *Preparation of Test Grass Lipid Sample and Lipid Standards*

The remainder of the test grass extract (~10 ml) was dried down under N at 50°C and re-suspended in 0.5ml $CHCl_3$. The SQDG, PI(Na^+), PS(Na^+), PG(Na^+), PE and PC lipid standards were sourced from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA) in powder form while the MGDG and DGDG lipid standards were sourced from Larodan (Solna, Sweden) in liquid form (dissolved in $CHCl_3$:MeOH). The purity of the lipid standards was not certified, but was assumed to be >95%. The powdered lipids were dissolved in $CHCl_3$ to an approximate concentration of 5 mg/ml. The lipids that were received in liquid were dried down at 50°C under N then re-dissolved in $CHCl_3$ to approximate concentrations of 5 mg/ml. The volumes of lipid standards spotted onto the TLC plate are stated in Table 7.1.

Table 7.1 Volumes of individual lipid standards spotted onto the first 2D-TLC standards plate

Lipid	2D-TLC	
	Vol. of standard spotted (µl)	**Equivalent amount of lipid (mg)***
MGDG	200	1.00
DGDG	150	0.75
PS (Na^+)	100	0.50
PC	100	0.50
PG (Na^+)	100	0.50
PE	100	0.50
SQDG	50	0.25
PI (Na^+)	50	0.25
Total	*850 µl*	*4.25 mg*

*based on the assumed concentration of 5 mg/ml.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyl-diacylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidyl-glycerol; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol; PI, phosphatidylinositol.

♦ *2D-TLC*

The TLC tank containing Solvent 1 was 'primed' for ~30 min with an adsorption pad. Meanwhile, the test grass extract and the lipid standards mixture were spotted onto separate 20cm x 20cm 1mm SIL-G/UV_{254} glass-backed TLC plates in the bottom, left-hand corner. The TLC plates were then placed onto a rack and into the development tank and left to develop. The run time for Solvent 1 was 4 hr 30 min. The TLC plates were then removed from the tank and allowed to dry for ~30min in the fume cupboard followed by ~30 min in a desiccator. After rotating the plates 90^o anti-clockwise, they were placed into the second TLC tank contained Solvent 2 which had already been 'primed' for ~30 min. The run time for Solvent 2 was 4 hr 20 min. After the second development, plates were removed from the tank and dried in a fume cupboard for ~30 min before transferring them to a desiccator to dry completely under vacuum overnight.

♦ *Visualising Lipid Fractions*

The plates were removed from the desiccator, sprayed with 2,7-dichlorofluorescin and allowed to develop in a dark room for ~20 min. Lipids were visualised using UV_{254} light and their positions lightly marked with a pencil. There was good separation of the lipids on both the test grass and lipid standards plates; however it was difficult to identify many of the lipids firmly due to 'smudging' of the spots, therefore it was decided not to proceed with removing and methylating the lipid spots. Subsequently, the plates were observed using a UV-A lamp in place of the UV_{254} and this resulted in better and more defined fluorescence of the lipids with less interference from background fluorescence.

♦ *Stage 2 Outcomes*

Figure 7.8 shows the resulting lipid separation and identification for the 'test grass' lipid sample and the lipid standard plates. The total run-time for this second attempt was over a third longer than the first attempt. Exact reasons for this longer runtime are unclear, however TLC is known to be sensitive to differences in temperature, pressure, humidity etc. The discovery that using a UV-A lamp to visualise the lipids gave much better and clearer fluorescence from the lipid spots was a great step forward. However, it was felt the runs-times on this occasion were too long, resulting in 'smudging' of the lipid fractions and thus preventing accurate identification of lipids. It was decided to run a second lipid standards plate and test whether leaving the adsorption pads, which are usually just used to 'prime' the TLC tanks by saturating the atmosphere, in the tanks while the plates are developing would help accelerate the run times and result in more defined separation of lipids.

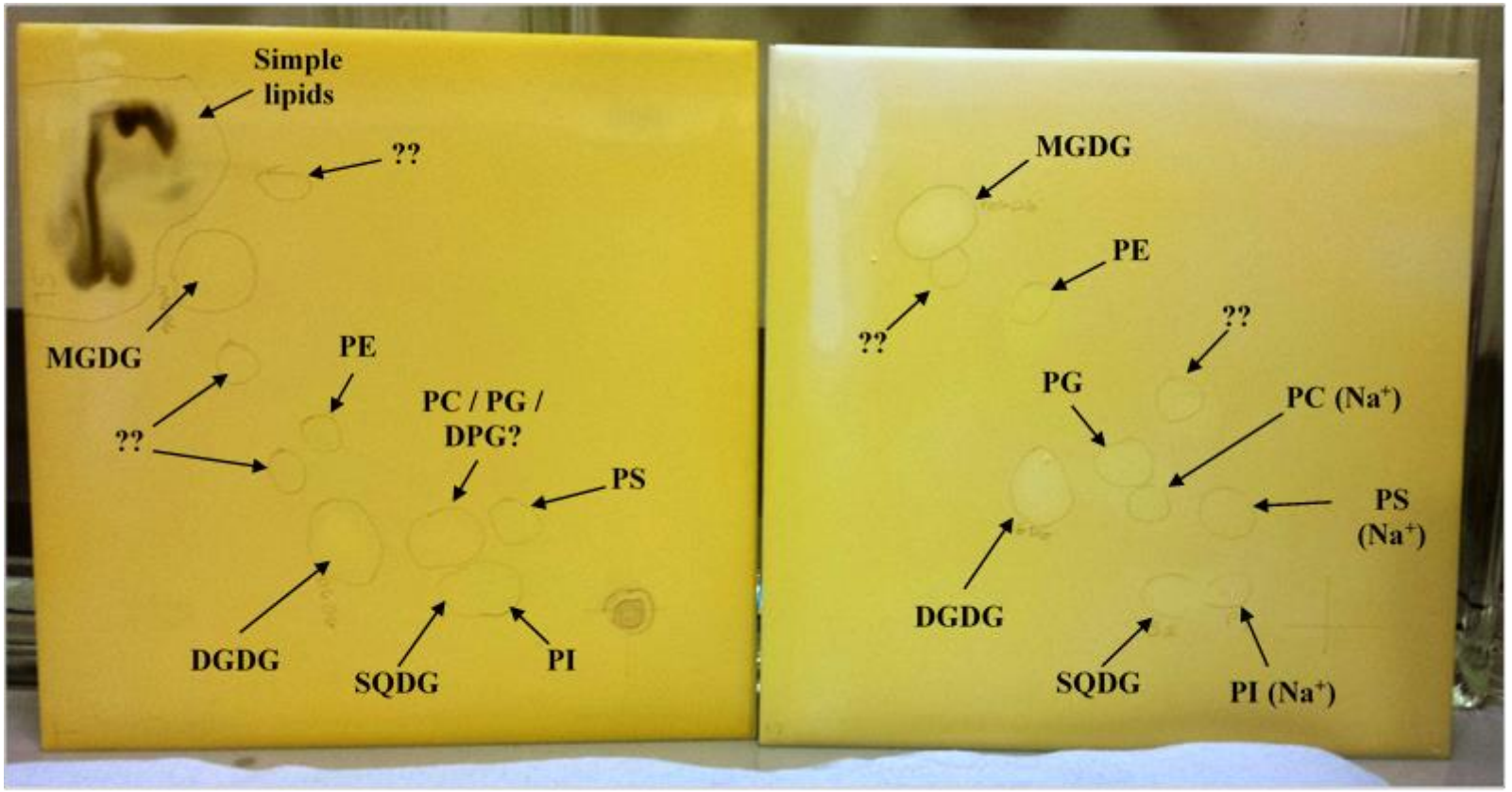

Figure 7.8 Resulting lipid separation following 2D-TLC for the test grass (left) and lipid standards (right) plates.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol (Cardiolipin); PE, phosphatidylethanolamine; ??, unknown.

Stage 3. Lipid Standards: Base Only Methylation

♦ *Preparation of Lipid Standards*

A new TLC plate was spotted with the lipid standards using the volumes stated in Table 7.2. It was decided to exclude the Na^+ salt lipids as it was thought that these would interact differently with the silica and eluent compared to the non-salt form (as found in grass).

Table 7.2 Volumes of individual lipid standards used to create the lipid mixture

	2D-TLC	
Lipid	**Volume spotted (µl)**	**Lipid equivalent (mg)***
MGDG	200	1.00
DGDG	150	0.75
PC	100	0.50
PE	100	0.50
SQDG	75	0.38
Total	*625 µl*	*4.13 mg*

*based on the assumed concentration of 5 mg/ml.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

♦ *2D-TLC*

The TLC tank containing Solvent 1 was 'primed' for ~30 min with an adsorption pad. Meanwhile, the lipid standards were spotted onto a 20cm x 20cm 1mm SIL-G/UV_{254} glass-backed TLC plate. It was then placed into the tank, which also contained adsorption pads at the back and sides of the tank, and allowed to develop. The run time for the first solvent was 2 hours 20 min. The plate was then removed from the tank and allowed to dry for ~30 min in the fume cupboard only. After rotating the plate 90^o anti-clockwise it was placed into the second pre-'primed' TLC tank containing Solvent 2, which also had adsorption pads at the back and sides of the tank.

During the second development, it was noticed that the silica started detaching from the glass-back, particularly where the plate was submerged in the eluent. However, the plate was allowed to complete the second development, which took 1 hr 50 min. After the second development, the plate was removed from the tank, dried in a fume cupboard for ~30 min then retained in a desiccator to dry completely under vacuum overnight.

♦ *Visualising and Removing Lipid Fractions*

The plate was removed from the desiccator, sprayed with 2,7-dichlorofluorescin and allowed to develop in a dark room for ~30 min. Lipids were visualised using a UV-A light and their positions lightly marked with a pencil. The lipid fractions were then scraped off and placed into individual culture tubes. A spatula was then used to break-up the silica into a fine powder.

♦ *Methylation*

A base-only methylation technique derived from Kramer *et al* (2001) was used in this instance. Base methylations work well for esterified FAs therefore it was believed this would be sufficient for methylating MGDG, DGDG, SQDG, PE and PC. One ml of heptane, containing 1.0 mg/ml heneicosanoic acid (C21:0) methyl ester as the internal standard (Sigma-Aldrich Co., USA), and 4 ml 0.5M sodium methoxide/MeOH was added to the tube. Samples were gently mixed and heated for 15 min at 50^{o}C in a water bath. Once cooled, 0.2 ml of glacial acetic acid, 2 ml n-heptane and 2 ml distilled water were added. Tubes were then vortexed and centrifuged at 2000 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube, to which a small spatula of anhydrous sodium sulphate added, mixed, left for 10 min then centrifuged as before. Gas Chromatography (GC) vials were then filled using a glass

Pasteur pipette and capped. Fatty acids were then quantified using GC-FID as described previously.

♦ *Stage 3 Outcomes*

Many issues were encountered during this stage. The reason for the silica detaching from the plate may be due to a poorly manufactured plate, or could be due to insufficient drying of the plate between the first and second development (~30 min fume cupboard only compared to the previous plates which were given ~30 min fume cupboard and ~30 min desiccator). Shorter run times were achieved by leaving adsorption pads in the tank. However the lipids did not to migrate as far up and across the plate compared with a longer run (see Figure 7.9) and although there was clear separation of MGDG there was little separation between the other lipids. This was not too much of an issue when using the lipid standards, as five separate spots relating to the individual lipids could be detected. However, it was felt that with a more complex matrix of lipids, such as that from grass, this level of separation would not be sufficient to enable confident separation and identification of lipids.

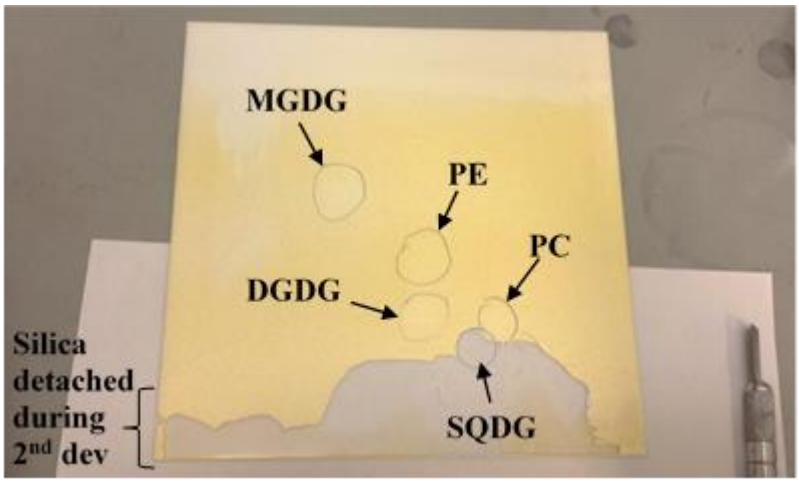

Figure 7.9 Lipid standards plate, which had a total run time of 3hr 10min, showing poor migration of lipids and where the silica detached from the plate.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine.

Furthermore, it was discovered that recovery of lipid from the silica was very poor, with lower recoveries for the galactolipids compared to the phospholipids. The calculated recoveries for each of the lipids are shown in Table 7.3. Speculated reasons for these poor recoveries were:

- Lipid fluorescence issues
  - Under-spraying of the plate would lead to the lipids not fluorescing well due to a lack of fluorescent marker
  - Over-spraying of plates may lead to false-negatives due to interference from background fluorescence.
- Imbalance in the amount of lipid vs. adsorbent (silica)
  - As a result of incorrect lipid loading
  - Christie and Han (2010) suggested the adsorbent to lipid ratio should be no higher than 4000:1.
- Incorrect methylation technique
- Speed of solvent runs

It was decided firstly to confirm the concentrations of the lipid standards via a direct methylation of the individual lipid standards along with a repeat of the 2D-TLC using the same volumes of lipid standards to enable a comparison between direct methylation vs. 2D-TLC recovery. Also, adequate drying time between first and second development would be ensured to avoid potential detachment of the silica from the plate. The effectiveness of an alternative fluorescent spray (Primuline) would be tested for visualising the lipids under UV-A lighting and the Sukhija and Palmquist (1988) methylation would be used for both the direct and 2D-TLC methylations.

Table 7.3 Calculated lipid recoveries after 2D-TLC and base-only methylation

Lipid	Amount loaded onto plate (mg)*	Amount recovered from plate (mg)	% Recovery
MGDG	1.00	0.54	54.37
DGDG	0.75	0.23	31.10
PC	0.50	0.34	68.14
PE	0.50	0.35	69.15
SQDG	0.38	0.06	15.66
Total	*4.13*	*1.52*	*36.80*

*based on lipid volumes and the assumed concentration of 5 mg/ml.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol.

Stage 4. Lipid Standards: Checking Concentrations and Recoveries

♦ *Preparation of Lipid Standards*

The volumes of each lipid standard used for the 2D-TLC and the direct methylation are given in Table 7.4.

Table 7.4 Volumes of lipid standards used for a comparison of 2D-TLC and direct methylation

Lipid	2D-TLC		Direct methylation	
	Volume spotted (µl)	Equivalent lipid (mg)*	Volume used (µl)	Equivalent lipid (mg)*
MGDG	200	1.00	200	1.00
DGDG	150	0.75	150	0.75
PC	100	0.50	100	0.50
PE	100	0.50	100	0.50
SQDG	50	0.25	50	0.25
Total	*625 µl*	*3 mg*	-	-

*based on the assumed concentration of 5 mg/ml.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol.

♦ *2D-TLC*

The TLC tank containing Solvent 1 was 'primed' for ~30 min with an adsorption pad. Meanwhile, the lipid standards were spotted onto a 20cm x 20cm 1mm SIL-G/UV_{254} glass-backed TLC plate which was then placed into the TLC tank with adsorption pads at the back and sides, and left to develop. The run time for the first solvent was 2 hr 15 min. The plate was then removed from the tank and allowed to dry for ~30 min in the fume cupboard and a further ~30 min in a desiccator. The plate was rotated 90^{o} anti-clockwise and placed into the second pre-'primed' TLC tank containing Solvent 2 and adsorption pads at the back and sides. Run time of Solvent 2 was 2 hr 45 min. The plate was then removed from the tank, dried in a fume cupboard for ~30 min and retained in a desiccator overnight to dry completely under vacuum.

♦ *Visualising and Removing Lipid Fractions*

The 2D-TLC plate was first sprayed with primuline and allowed to develop in a dark room for ~30 min. The plate was then examined under UV-A light to observe the lipids. Moderate fluorescence from the lipids was accomplished using the primuline agent, however separation and definition of the lipid spots was not ideal. Whether this was due to the reagent itself or some other contributing factors was uncertain, however it was decided to re-spray and develop the plate using the 2,7-dichlorofluorescein and observe under the UV-A light again. Indeed, the 2,7-dichlorofluorescein gave more defined lipid fluorescence however the separation of these lipids was still not ideal but satisfactory for identifying the five individual lipids. The position of the lipids was marked lightly with a pencil then scraped off and placed into individual culture tubes before crushing with a spatula to break up the silica.

♦ *Methylation (2D-TLC Fractions and Direct)*

The methylation procedure of Sukhija and Palmquist (1988), as described earlier with a 2 hr methylation at 70^{o}C, was used for both the direct and the 2D-TLC fractions, with quantification of the FAMEs done via GC-FID.

♦ *Stage 4 Outcomes*

The primuline did not achieve improved fluorescence and accordingly identification of the lipid spots was not improved. Also, the separation of the spots was similar to the previously run standard plate; which as explained earlier, was acceptable for separating the lipid standards but was considered unsuitable for separating a complex lipid matrix such as from grass. The poor migration and separation may be due to the presence of adsorption pads at the back and sides of the TLC tanks. Although this achieved the aim of reducing the run times of the solvents, it was feared that the run times had in actual fact become too quick. However, there is a fine balance between developing the plates too quickly, resulting in poor separation and migration of the lipids, versus running the plates too slowly which resulting in 'smudging' of the lipids. It was decided to only have adsorption pads present at either end of the TLC tanks in the hope to achieve this balance. A disappointing outcome of this stage was still finding poor recoveries of the lipids even when the Sukhija and Palmquist (1988) methylation was used (see Table 7.5), since similar recoveries were found to those of the base-only methylation; with the exception of SQDG which is an assumed vs. actual lipid standard concentration (>60% difference) issue rather than improved recovery. The assumed concentrations of the DGDG was also inaccurate with ~40% difference between expected and actual concentration. The PC and PE concentrations were much closer to the expected value but still had 20% and 14% difference, respectively. There may still be an issue with the amount of lipid being loaded onto these plates, resulting

in a poor lipid to adsorbent ratio which may in turn be affecting the attainable recoveries.

Moving forward, it was decided to try using thinner 250µm plates, which were already available in the laboratory, to test whether a) thinner plates would lead to quicker run time relative to 1mm plates, and b) it was possible to achieve improved recoveries due to the reduced amount of adsorbent relative to the amount of lipid. Additionally, two plates would be run with differing total lipid loadings to investigate this issue further.

Table 7.5 Lipid recoveries from the direct methylation and 2D-TLC

Lipid	Expected[1] (mg)	Actual[2] (mg)	% difference	Fractionated[3] (mg)	% recovery[*]
MGDG	1.00	1.07	6.79	0.42	39.47
DGDG	0.75	0.44	-41.81	0.24	55.80
PC	0.50	0.40	-20.26	0.31	76.88
PE	0.50	0.43	-14.79	0.34	78.77
SQDG	0.38	0.14	-62.22	0.09	60.21

[1]Based on the assumed concentrations of 5 mg/ml; [2]Amount of lipid recovered from the direct methylation; [3]Amount of lipid recovered from the 2D-TLC; [*]Recovery of lipid after 2D-TLC relative to the direct methylation results.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyldiacylglycerol.

Stage 5. Lipid Standards: Using Thinner Plates

♦ *Preparation of Lipid Standards*

The volumes of each lipid standard used for the thinner 250µm TLC plates with 'high' and 'low' lipid loadings are given in Table 7.6. The SQDG standard was not used because of the disagreement between the expected vs. the direct methylation results and because the bulk volume of this standard was beginning to get low.

Table 7.6 'High' and 'low' lipid loadings on the thinner 2D-TLC plates

Lipid	2D-TLC 'high'			2D-TLC 'low'		
	Volume spotted (µl)	Lipid[1] (mg)	Lipid[2] (mg)	Volume used (µl)	Lipid[1] (mg)	Lipid[2] (mg)
MGDG	200	1.00	1.07	100	0.50	0.54
DGDG	150	0.75	0.44	75	0.38	0.22
PC	100	0.50	0.40	50	0.25	0.20
PE	100	0.50	0.43	50	0.25	0.22
Total	*550 µl*	*2.75 mg*	*2.34 mg*	*275 µl*	*1.38 mg*	*1.18 mg*

[1]Based on assumed concentration of 5 mg/ml; [2]Based on concentrations derived from direct methylation.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

♦ *2D-TLC*

The TLC tank containing Solvent 1 was 'primed' using an adsorption pad for ~30 min. In the meantime, two 20cm x 20cm 250µm POLYGRAM SIL-G/UV_{254} polyester-backed TLC plates (HiChrom Ltd, Reading, Berkshire, UK) were loaded with the lipid loadings described in Table 7.6 in the bottom left-hand corner. The plates were then placed into the TLC tank with adsorption pads at the sides only and allowed to develop. The run time of the first development was 2 hr 50 min. The plates were then removed from the tank and dried for ~30 min in the fume cupboard followed by ~30 min in a desiccator. Plates were then rotated 90^{o} anti-clockwise and placed into the second pre-primed TLC tank containing Solvent 2, with adsorption pads at the sides only, for the second development. The run time of Solvent 2 was 2 hr 35 min. The plates were removed from the tank, air-dried in a fume cupboard for ~30 min then transferred to a desiccator and allowed to dry completely under vacuum overnight.

♦ *Stage 5 Outcomes*

Plates were not scraped and methylated because the polyester backing reacted with the $CHCl_3$ in the solvent mixtures. Also during this time, contact was made with Dr. Christie regarding this procedure requesting advice on how to improve our current method. He stated that the 2 hr methylation at 70^{o}C was far too short and suggested an overnight methylation at 50^{o}C would be more appropriate. Therefore it was decided to repeat the same lipid loadings using the glass-backed plates and use the overnight methylation to see whether this would improve recoveries from the 1mm thick TLC plates. A direct methylation would also be repeated using the higher volumes of the individual lipids.

Stage 6. Lipid Standards: Overnight Methylation and Recoveries

♦ *Preparation of Lipid Standards*

The same volumes of lipid standards stated in Table 7.6 were spotted onto two separate 1mm glass-backed TLC plates.

♦ *2D-TLC*

The first TLC tank was 'primed' using an adsorption pad for ~30 min. Meanwhile, lipid standards were spotted onto the 20cm x 20cm 1mm SIL-G/UV_{254} glass-backed TLC plates. The plates were then placed into the TLC tank, which had adsorption pads at either end of the tank only, and left to develop. The run time of Solvent 1 was 2 hr 55 min. Plates were then removed from the tank and placed in a fume cupboard to dry for ~30 min then transferred to a desiccator to dry for a further ~30 min. The second TLC tank was primed for ~30 min with an adsorption pad. The dried plates were rotated 90^{o} anti-clockwise then placed into the second tank, again with adsorption pads

at the sides only, and left to development. The run time of solvent 2 was 3 hr 20 min. The plates were removed from the tank, air-dried in a fume cupboard for ~30 min then transferred to a desiccator and allowed to dry completely under vacuum overnight.

♦ *Visualising and Removing Lipid Fractions*

The plates were removed from the desiccator, sprayed with 2,7-dichlorofluorescin and allowed to develop in a dark room for ~30 min. Lipids were visualised using a UV-A light and their positions lightly marked with a pencil. These were then scraped off and placed into individual culture tubes. The silica in each tube was then broken up into a fine powder using a spatula.

♦ *Methylation*

The Sukhija and Palmquist (1988) methylation procedure was followed, however a 16 hr (overnight) methylation at 50^{o}C was used in place of the normal 2 hr methylation, as suggested by Dr. Christie.

♦ *Stage 6 Outcomes*

The direct methylation results were similar to those reported earlier in this chapter (Table 7.5), confirming the specific concentrations of these lipid standards. The recoveries between the 'high' and 'low' lipid loading were comparable with each other, especially for the phospholipid which achieved over 93% recoveries (see Table 7.7). The recoveries of the galactolipids were slightly more variable between the 'high' and 'low' lipid loadings, with better recovery of MGDG on the 'high' plate while better recovery of DGDG was seen on the 'low' plate. Nonetheless, recoveries of all lipids were improved with the overnight methylation compared to the base-only Kramer *et al* (2001) methylation and the 2 hr at 70^{o}C Sukhija and Palmquist (1988) methylation, as shown in Table 7.8.

Table 7.7 Comparison of results and recoveries from the higher lipid loading vs. the lower lipid loading on the 1mm thick TLC plates

	'high' loading			'low' loading		
Lipid	**Direct (mg)**	**2D-TLC (mg)**	**% recovery**	**Direct* (mg)**	**2D-TLC (mg)**	**% recovery**
MGDG	1.07	0.92	86.19	0.54	0.42	78.76
DGDG	0.41	0.27	64.36	0.21	0.14	70.08
PC	0.45	0.42	94.73	0.22	0.21	94.18
PE	0.42	0.40	93.83	0.21	0.20	95.01

*estimated by halving the direct methylation results of the higher volumes of lipids.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table 7.8 Comparison of lipid recoveries (%) between the base-only, 2 hr and overnight (16 hr) methylation procedures

	% recovery			
			Sukhija	
Lipid	**Kramer**	**Sukhija**	***16hr, 50°C***	
	Base-only	***2hr, 70°C***	**'high'**	**'low'**
MGDG	54.37	39.47	86.19	78.76
DGDG	31.10	55.80	64.36	70.08
PC	68.14	76.88	94.73	94.18
PE	69.15	78.77	93.83	95.01

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

The recoveries achieved for the phospholipids are quite acceptable, however the recoveries of the galactolipids are less so. In the likelihood that 100% recoveries are not achievable, all the lipids should ideally have equivalent recoveries in order to enable direct comparison between the proportions of the lipid fractions. Further work is needed to achieve at best similar recoveries of the galactolipids relative to the phospholipids. Also, this work has largely only been carried out using lipid standards. Lipid extracted from grass is much more complex and includes other constituents such as chlorophyll which may interfere with the lipid separation. In light of the number of issues still to be resolved, a search for an alternative means to explore the original hypothesis was carried out.

7.3.4 Solid Phase Extraction (SPE)

A solid phase extraction (SPE) method was found which separated total lipid into three separate fractions, namely neutral (NL), galactolipid (GL) and phospholipid (PL), using 10 ml $CHCl_3$ with 1% acetic acid (AA), 15ml Acetone : MeOH (9:1) and 10ml MeOH to elute these lipid fractions, respectively (Yao and Rastetter, 1985). Although this method would result in less detail and information regarding the lipid composition of the experimental grass samples, it is sufficient for investigating the original hypothesis.

Stage 1. Test Grass: Familiarising with the Method

♦ *Test Grass Lipid Sample Preparation*

Approximately 1.0 g of a test grass sample was weighed into a culture tube, with the exact weight recorded. Ten ml of $CHCl_3$: MeOH (2:1) was added to the tube and swirled. The tube was then placed on an orbital shaker and shaken at ~300 rpm for 5 min followed by centrifugation at 1500 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube. This process was repeated a further two times giving a total extract volume of approximately 30 ml.

Five ml of this extract was then transferred to a new culture tube using a pipette and dried down under N at 50^{o}C. The lipid (~5 mg) was then re-suspended in 1 ml $CHCl_3$.

♦ *SPE*

A Strata SI-1 Silica (55um, 70A, 500mg/3ml) Teflon coated SPE cartridge (Phenomenex, Macclesfield, Cheshire, UK) was set up on a SPE vacuum manifold with a three-way stopcock and pump attached. The SPE cartridge was primed by filling it with ~3 ml of $CHCl_3$ which was allowed to completely drain through into a culture

tube to rinse the column. A second ~3 ml of $CHCl_3$ was added and this was allowed to drain through until the solvent level dropped to the top of the gel, at which point the stopcock was turned off. A fresh culture tube was placed into the tank under the SPE cartridge and the test grass lipid sample (~1 ml) was transferred to the top of the silica gel using a Pasteur pipette. After complete elution of the conditioning solvent, 10 ml of $CHCl_3$ was carefully added to the column in quantities of 2.5 ml and the pressure in the tank adjusted to achieve a flow rate of ~1 ml/min. This elutes the NL fraction. After draining of the first solvent was complete, new culture tubes were placed into the tank and 15 ml of Acetone : MeOH (9:1) was added to the column (in quantities of 2.5 ml) to elute the GL fraction. Finally, after complete draining of the second solvent, a third set of culture tubes were placed into the tank and 10 ml of MeOH added to the column (4 x 2.5 ml) to elute the PL fraction. It is important to note that although eluents are drained off completely between fractions, the silica gel phase is not allowed to dry as this may cause problems with subsequent elutions of lipid fractions.

♦ *Methylation*

Fractions were dried down at 50^{o}C under N. Methylation was then carried out following the Sukhija and Palmquist (1988) procedure, using C21:0 methyl ester (Sigma-Aldrich Co.,USA) in toluene (0.5 mg/ml) as the internal standard and the customary 2 hr methylation at 70^{o}C. Fatty acids were then quantified using GC-FID following the conditions described earlier.

♦ *Stage 1 Outcomes*

The main aim and outcome of this stage was to familiarise with the method and to establish the approximate length of time needed to complete the method. The one cartridge took approximately an hour in total to run, with a possibility of running a

maximum of eight cartridges simultaneously. However it was felt that four cartridges would be adequate to enable good control and supervision of the method. Additionally, with further appraisal of the literature, is was found that using $CHCl_3$ containing 1% AA during the first elution resulted in improved recovery of the NL fraction, particularly the FFAs. Moving forward, a direct methylation of lipid standards would be carried out, accompanied by comparison of $CHCl_3$ with and without the 1% AA on recovery of the NL fraction.

Stage 2. Lipid Standards: Method Adjustment and Lipid Recoveries

♦ *Lipid Standard Preparation*

Three lipid standard mixtures containing GL, PL and NL were created using the volumes of individual lipid standards as stated in Table 7.9. One would be used for direct methylation while the other two would undergo SPE to compare the effectiveness of $CHCl_3$ with 1% AA versus $CHCl_3$ only for eluting the NL fraction.

Table 7.9 Volumes of individual lipid standards used to create a lipid mixture which included neutral lipids (NL), galactolipids (GL) and phospholipids (PL)

Fraction	Lipid	Volume (µl)
GL	MGDG	600
	DGDG	450
PL	PE	300
	PC	300
NL	TAG	10
	DAG	10
	FFA	10
	Total	*1680 µl*

Abbreviations: GL, galactolipid; MGDG, monogalactosyl-diacylglycerol; DGDG, digalactosyldiacylglycerol; PL, phospho-lipid; ; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NL, neutral lipid; TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acid.

♦ *SPE*

Two Strata SI-1 Silica (55um, 70A, 500mg/3ml) Teflon coated SPE cartridge (Phenomenex, Macclesfield, Cheshire, UK) were set up on an SPE vacuum manifold with three-way stopcocks and pump. Cartridges were primed as described earlier and the solvent level dropped to the top of the gel. Lipid standard mixtures (as described in Table 7.9) were introduced into each cartridge. After complete elution of the conditioning solvent, 10 ml of $CHCl_3$ was carefully added to one of the columns while 10 ml of $CHCl_3$ with 1% AA was added to the other (in quantities of 2.5 ml) to elute the NL fraction. The pressure was adjusted appropriately to achieve a flow rate of ~1 ml/min. The GL fraction was eluted with 15 ml of Acetone : MeOH (9:1) and the PL fraction with 10 ml of MeOH for both cartridges.

♦ *Methylation*

Fractions were dried down at 50^{o}C under N. Methylation was then carried out following the Sukhija and Palmquist (1988) procedure and FAMEs quantified using GC-FID as described earlier.

♦ *Stage 2 Outcomes*

The results and recoveries for the direct methylation and the $CHCl_3$ excluding and including the 1% AA are shown in Table 7.10. Inclusion of the 1% AA did indeed result in improved recovery of the NL fraction (64% vs. 111% recovery). Also, overall recoveries of the fractions were much higher and more consistent compared to the recoveries achieved with the 2D-TLC method. Accordingly, it was decided to proceed with this method and carry out the analysis on the experimental grass samples using $CHCl_3$ containing 1% AA for the first elution.

Table 7.10 Results and recoveries from the direct methylation and the chloroform without and with 1% acetic acid

Lipid Fraction	Direct Methylation	SPE			
		$CHCl_3$ (-)[1]	% Recovery	$CHCl_3$ (+)[2]	% Recovery
NL	0.65	0.42	64.36	0.72	111.04
GL	4.35	4.48	102.95	4.26	97.76
PL	2.67	2.61	97.70	2.58	96.61

[1]Chloroform without acetic acid; [2]Chloroform with 1% acetic acid.
Abbreviations: NL, neutral lipid; GL, galactolipid; PL, phospholipid.

Stage 3. Experimental Grass Samples

♦ ***Extraction* and *Preparation of Experimental Grass Lipid Samples***

For each of the 24 composite grass samples described earlier in section 6.2.1 approximately 1.0 g of sample was weighed into a culture tube, and exact weight recorded. Ten ml of $CHCl_3$:MeOH (2:1) was added to the tube, swirled, then placed on an orbital shaker and shaken at ~300 rpm for 5 min followed by centrifugation at 1500 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube. This process was repeated a further two times using 7 ml and 5 ml of CHCl3:MeOH (2:1), giving a total extract volume of approximately 22 ml.

Five ml of the extracts were then transferred into new culture tubes using a pipette and dried down under N at 50^{o}C. The lipid was then re-suspended in 1.0 ml $CHCl_3$ and used for SPE. Additionally, another 5 ml of the extracts were transferred into new culture tubes using a pipette and used for direct methylation to determine total lipid (TL) and to calculate recoveries from the SPE.

♦ *SPE*

Four Strata SI-1 Silica (55um, 70A, 500mg/3ml) Teflon coated SPE cartridges (Phenomenex, Macclesfield, Cheshire, UK) were set up on an SPE vacuum manifold with three-way stopcocks and pump. Cartridges were primed using $CHCl_3$, as described earlier, and a grass lipid sample loaded into each cartridge. After complete elution of the conditioning solvent, 10 ml of $CHCl_3$ containing 1% AA was carefully added to the columns, followed by 15 ml Acetone : MeOH (9:1) and finally 10 ml MeOH to elute the NL, GL and PL fractions, respectively. This process was repeated until all twenty-four samples had been fractionated.

♦ *Methylation*

The SPE fractions and the 5 ml sub-samples of extract for direct methylation were dried down at 50^{o}C under N then methylated following the procedure of Sukhija and Palmquist (1988) with a 2 hr methylation at 70^{o}C. Fatty acids were quantified using GC-FID.

♦ *Stage 3 Outcomes*

Table 7.11 shows the sum of the fractions after separation of the lipid extract via SPE along with the total lipid recovered via the direct methylation and the calculated recoveries (SPE vs. direct methylation). The overall average recovery of lipid from SPE was 86%, ranging from 82% to 90%. These recoveries were lower than expected, considering >95% recoveries were achieved with the lipid standard mixtures. This may be due to the grass lipid extract being a more ‘complex’ matrix which includes components such as chlorophyll and small, fibrous fragments of grass, and these extra components may have interfered with the elution of lipids from the column. Also, running four SPE cartridges at a time meant flow rates in each cartridge varied, due to

some samples flowing better than others – possibly due to variable lipid content or other interfering factors. It was decided to investigate whether running the SPE cartridges individually, and thus having better control over the eluent flow rates, would improve lipid separations and recoveries. This would be carried out on a set of four randomly selected samples.

Additionally, doubt was cast on whether the original 22ml $CHCl_3$: MeOH (2:1) extraction had managed to completely extract all the lipid from the freeze-dried composite grass samples. This concern arose whilst comparing calculations of the concentrations of the 22ml extracts (and from this the amount of lipid extracted from the 1 g composite samples) against the TFA genotype averages generated from the original FA compositional data presented in Chapter 5 and with the test grass 30ml extract used in for the first stage of SPE method development (see Table 7.12 and Table 7.13). As a result, the $CHCl_3$: MeOH (2:1) extractions of the composite grass samples were repeated using a total extract volume of 30ml rather than 22ml.

Table 7.11 Calculation of lipid recovery (%) from SPE lipid separation vs. direct methylation

Sample No.	Total FAME (mg) in 5ml extract		% Recovery
	Sum of SPE Fractions (NL + GL + PL)	Direct Methylation (TL)	
01	3.60	4.31	83.57
02	4.26	5.13	83.09
03	5.60	6.68	83.77
04	5.44	6.51	83.47
05	5.62	6.70	83.85
06	3.75	4.38	85.67
07	3.78	4.55	83.11
08	3.69	4.28	86.15
09	4.50	5.25	85.82
10	4.41	4.90	89.85
11	4.35	4.91	88.56
12	4.57	5.19	88.10
13	4.33	5.03	86.13
14	4.31	5.15	83.82
15	4.54	5.10	89.13
16	4.58	5.25	87.23
17	4.04	4.57	88.41
18	5.33	6.20	85.93
19	4.12	4.95	83.32
20	4.21	4.90	85.85
21	3.51	4.16	84.57
22	3.76	4.46	84.31
23	3.58	4.35	82.48
24	4.61	5.22	88.29
Average	*4.35*	*5.09*	*85.60*

Abbreviations: NL, neutral lipid; GL, galactolipid; PL, phospholipid; TL, total lipid.

Table 7.12 Calculated lipid recovery from the freeze-dried experimental grass samples using a 22ml $CHCl_3$: MeOH (2:1) extraction

Sample No.	TL in 5ml[1]	TL in 22ml (total extract vol.)	TL extracted from 1g[2]	TFA mg/g DM[3]	approx. % lipid recovery
01	4.31	18.97	18.92	26.20	72.42
02	5.13	22.56	22.62	29.92	75.41
03	6.68	29.39	29.40	35.66	82.43
04	6.51	28.66	28.72	34.75	82.47
05	6.70	29.50	29.42	35.81	82.37
06	4.38	19.27	19.18	26.91	71.60
07	4.55	20.02	19.97	26.23	76.33
08	4.28	18.83	18.81	27.12	69.44
09	5.25	23.08	23.01	30.12	76.64
10	4.90	21.57	21.58	28.26	76.33
11	4.91	21.61	21.57	28.12	76.86
12	5.19	22.83	22.75	31.00	73.63
13	5.03	22.12	22.16	28.14	78.59
14	5.15	22.65	22.69	28.44	79.64
15	5.10	22.44	22.34	29.25	76.71
16	5.25	23.10	23.14	25.38	91.00
17	4.57	20.11	20.19	28.95	69.46
18	6.20	27.28	27.17	34.34	79.44
19	4.95	21.77	21.73	27.36	79.56
20	4.90	21.56	21.46	28.11	76.70
21	4.16	18.28	18.35	26.25	69.66
22	4.46	19.64	19.58	27.59	71.18
23	4.35	19.12	19.12	24.52	77.97
24	5.22	22.95	22.93	27.54	83.33
Average:	*5.09*	*22.39*	*22.37*	*29.00*	*77.05*

[1]By direct methylation of extract; [2]Corrected for actual grass sample weight; [3]Genotype average as calculated from ANOVA analysis of replicate FA compositional data in Chapter 5.
Abbreviations: TL, total lipid; TFA, total fatty acid.

Table 7.13 Calculated lipid recovery for the test grass sample which was extracted using 30 ml $CHCl_3$: MeOH and fractionated individually using solid phase extraction (SPE)

Sample ID	TL in 5ml[1]	TL in 30ml (total extract vol.)	TL extracted from 1g[2]	TFA mg/g DM[3]	approx. % lipid recovery
TEST GRASS	4.49	26.93	26.92	28.58	94.21

[1]Sum of fractions (NL+GL+PL); [2]Corrected for actual grass sample weight; [3]Direct methylation of freeze-dried sample.
Abbreviations: TL, total lipid; TFA, total fatty acid.

Stage 4. Sample repeats: SPE individually vs. groups of four

♦ *Sample Preparation*

Five ml of lipid extract from four randomly selected experimental samples was dried down under N at 50^{o}C then re-suspended in 1.0 ml $CHCl_3$ for SPE separation.

♦ *SPE*

A single SPE cartridge was set up on an SPE vacuum manifold with a three-way stopcock and pump. The cartridge was primed using $CHCl_3$, as described earlier, and the lipid sample loaded into the cartridge. After complete elution of the conditioning solvent, 10 ml of $CHCl_3$ containing 1% AA was carefully added to the column, followed by 15 ml Acetone : MeOH (9:1) and finally 10 ml MeOH to elute the NL, GL and PL fractions, respectively. This process was repeated until all samples had been fractionated.

♦ *Methylation*

The SPE fractions were dried down at 50^{o}C under N then methylated using the procedure of Sukhija and Palmquist (1988) with a 2 hr methylation at 70^{o}C followed by quantification of FAMEs by GC-FID.

♦ *Stage 4 Outcomes*

Calculated recoveries are shown in Table 7.14 and Table 7.15. Running the samples individually on the SPE vacuum manifold resulted in a 7% increase in average lipid recovery compared to running the samples in groups of four (92% vs. 85%). These improved recoveries, although not equal to, are at least closer to the recoveries achieved with the lipid standards. Also, the proportions of the lipid fractions of individually fractionated samples were more akin to what was expected.

Table 7.14 Comparison of the lipid recoveries when carrying out solid phase extraction (SPE) in groups of four versus individually

	SPE (x 4)			SPE (x 1)		
Sample No.	**Sum of fractions (NL+GL+PL)**	**Direct methylation (TL)**	**% Recovery**	**Sum of fractions (NL+GL+PL)**	**Direct methylation (TL)**	**% Recovery**
01	3.60	4.31	83.56	4.07	4.31	94.32
09	4.50	5.25	85.82	4.79	5.25	91.33
18	5.33	6.20	85.93	5.69	6.20	91.70
22	3.76	4.46	84.31	4.11	4.46	92.17
Average	*4.30*	*5.06*	*84.90*	*4.66*	*5.06*	*92.38*

Abbreviations: NL, neutral lipid; GL, galactolipid; PL, phospholipid; TL, total lipid.

Table 7.15 Comparison of the proportions of the lipid fractions when carrying out solid phase extraction (SPE) in groups of four versus individually

	SPE (x 4)			SPE (x 1)		
Sample No.	**GL%**	**NL%**	**PL%**	**GL%**	**NL%**	**PL%**
01	46.59	41.37	12.04	65.24	22.57	12.19
09	57.79	30.44	11.76	72.98	15.16	11.86
18	75.09	15.56	9.35	75.31	15.06	9.63
22	70.74	18.26	11.00	70.92	18.19	10.88
Average	*62.55*	*26.41*	*11.04*	*71.11*	*17.74*	*11.14*

Abbreviations: NL, neutral lipid; GL, galactolipid; PL, phospholipid; TL, total lipid.

Stage 5. *Repeated Experimental grass samples (Final method)*

♦ *Extraction* and *Preparation of Lipid Samples*

Approximately 1.0 g of each of the composite grass samples was weighed into culture tubes, with the exact weight recorded. Ten ml of $CHCl_3$: MeOH (2:1) was added to the tube, swirled, then placed on an orbital shaker and shaken at ~300 rpm for 5 min followed by centrifugation at 1500 rpm for 5 min. A glass Pasteur pipette was used to transfer the top layer into a second culture tube. This process was repeated a further two times, giving a total extract volume of approximately 30 ml. For composite grass samples where less than 1 g was available, weights and total extract volume were adjusted accordingly to achieve the same ratio of sample to $CHCl_3$: MeOH.

Five ml of the extracts were then transferred into new culture tubes using a pipette and dried down under N at 50^{o}C. The lipid was then re-suspended in 1.0 ml $CHCl_3$ and used for SPE. Additionally, another 5 ml aliquot of each of the extracts was transferred into new culture tubes using a pipette and used for direct methylation to determine total lipid (TL) and to calculate recoveries from the SPE. Smaller volumes were used for direct methylation where less than 1 g of composite sample was used for $CHCl_3$: MeOH (2:1) lipid extraction.

♦ *SPE*

Each lipid extract sample was fractionated individually. A single Strata SI-1 Silica (55um, 70A, 500mg/3ml) Teflon coated SPE cartridge (Phenomenex, Macclesfield, Cheshire, UK) was set up on an SPE vacuum manifold with a three-way stopcock and pump attached. The cartridge was primed using $CHCl_3$, as described earlier, followed by loading of the grass lipid sample into the cartridge. After complete elution of the

conditioning solvent, 10 ml of $CHCl_3$ containing 1% AA was carefully added to the column, followed by 15 ml Acetone : MeOH (9:1) and finally 10 ml MeOH to elute the NL, GL and PL fractions, respectively. This process was repeated until all four samples had been fractionated.

♦ *Methylation*

Lipid fractions resulting from the SPE procedure and the extract sub-samples for direct methylation were dried down at 50^{o}C under N then methylated via the Sukhija and Palmquist (1988) method with a 2 hr methylation at 70^{o}C. Fatty acids were then quantified using GC-FID.

♦ *Stage 5 Outcomes*

The approximate lipid recoveries for the 30 ml extractions are presented in Table 7.16. Unfortunately, using 30 ml of $CHCl_3$: MeOH (2:1) to extract the lipid from the sub-samples of the experimental grass composites did not result in improved extraction and recovery compared to the 22 ml extraction. However, a high R^2 value of 0.86 was found between average genotype TFA content (mg/g DM) as calculated from ANOVA analysis of replicate FA compositional data and the calculated total lipid extracted from 1g of composite sample (see Figure 7.10).

Nonetheless, running the samples individually resulted in improved lipid recoveries compared to running samples in groups of four, averaging at 92% compared to 86%, respectively (see Table 7.17). Due to time and resource constraints, the results from this method development stage were deemed adequate to enable investigation of the original hypothesis.

Table 7.16 Estimated lipid recovery from the freeze-dried experimental grass samples using a 30ml $CHCl_3$: MeOH (2:1) extraction

Sample No.	TL extracted from 1g[1]	TFA (mg/g DM)[2]	Approx. % lipid recovery
01*	18.96	26.20	72.38
02*	21.58	29.92	72.14
03	27.75	35.66	77.82
04	25.77	34.75	74.15
05	27.74	35.81	77.48
06	18.69	26.91	69.45
07	19.62	26.23	74.81
08	18.22	27.12	67.17
09	21.93	30.12	72.80
10	21.60	28.26	76.42
11	20.88	28.12	74.26
12	22.74	31.00	73.36
13	21.19	28.14	75.31
14	21.27	28.44	74.78
15	21.92	29.25	74.94
16	21.83	25.38	86.00
17	20.21	28.95	69.82
18	27.08	34.34	78.86
19	20.35	27.36	74.37
20	21.50	28.11	76.50
21	17.96	26.25	68.42
22	18.92	27.59	68.56
23	17.64	24.52	71.94
24	21.93	27.54	79.64
Average:	*21.55*	*29.00*	*74.22*

*Corrected for extracted sub-samples of <1g; [1]Corrected for actual grass sample weight; [2]Genotype average as calculated from ANOVA analysis of replicate FA compositional data in Chapter 5. Abbreviations: TL, total lipid; TFA, total fatty acids.

Table 7.17 Sum of total lipid (TL) from the SPE fractions, TL from the direct methylation and the calculated lipid recoveries (SPE vs. direct methylation)

Sample No.	Sum of fractions (NL + GL + PL)	Direct methylation (TL)	% Recovery
01	3.08	3.16	97.54
02	3.41	3.60	94.67
03	4.21	4.63	90.90
04	4.05	4.29	94.41
05	4.34	4.62	93.79
06	2.90	3.12	93.17
07	3.08	3.27	94.21
08	2.82	3.03	92.88
09	3.30	3.65	90.26
10	3.35	3.60	93.09
11	3.07	3.48	88.27
12	3.38	3.79	89.06
13	3.19	3.53	90.35
14	3.17	3.54	89.45
15	3.35	3.65	91.57
16	3.26	3.64	89.76
17	2.93	3.37	87.16
18	4.08	4.51	90.34
19	3.21	3.39	94.62
20	3.27	3.58	91.37
21	2.71	2.99	90.44
22	2.90	3.15	92.09
23	2.62	2.94	88.95
24	3.30	3.65	90.38
Average:	*3.29*	*3.59*	*91.61*

Abbreviations: NL, neutral lipid; GL, galactolipid; PL, phospholipid; TL, total lipid.

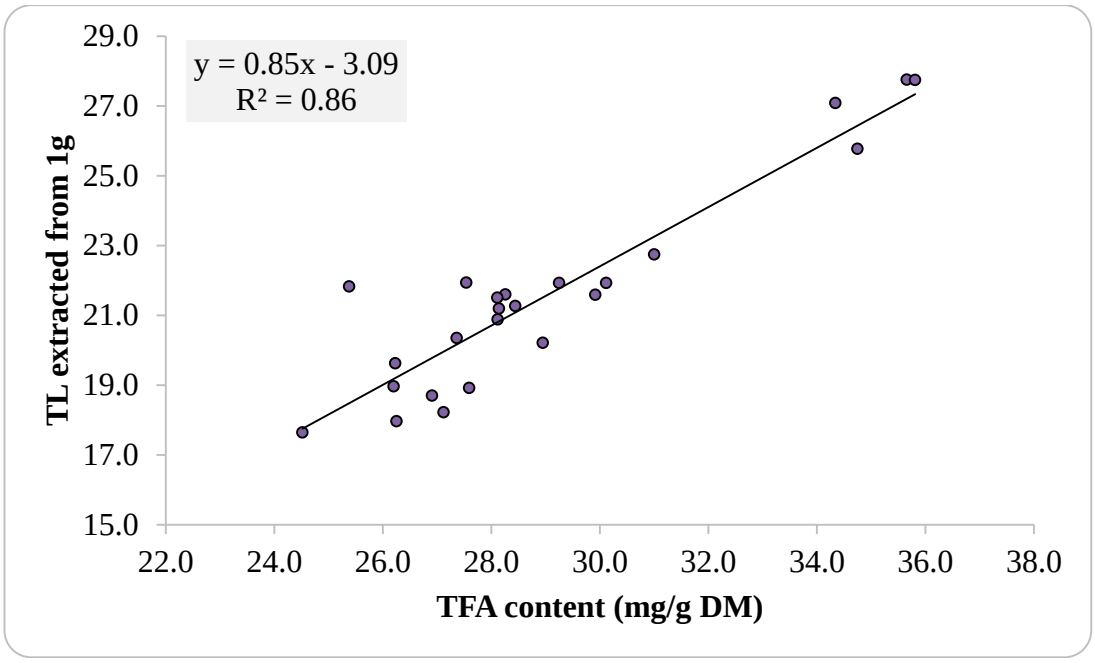

Figure 7.10 Relationship between mean genotype total fatty acid (TFA) content of four replicates (mg/g DM) relative to total lipid (TL) extracted from 1g of composite sample

7.3.5 Statistical Analysis

Genotype averages, as calculated from ANOVA analysis of replicate total chlorophyll and FA compositional data in Chapter 5, were used to quantify the relationships between chlorophyll, TFA and lipid proportions. Spearman's rank correlation and scatter plot matrices were generated using R software (version 3.2.1 for Windows, https://www.r-project.org/). Correlations were calculated between (a) total chlorophyll, TFA, POL%, FFA%, TAG% and DAG% derived from the 1D-TLC lipid separation, and (b) total chlorophyll, TFA, GL%, NL% and PL% derived from the SPE lipid separation.

7.4 Results

7.4.1 1D-TLC

The 1D-TLC method resulted in a polar lipid (POL) fraction, which included both galactolipids and phospholipids, and three neutral lipid fractions (FFA, TAG and DAG). The POL fraction ranged from 83.2% to 90.4%, with an across genotype average of 86.1%. Similar proportions of FFA and DAG were found across the genotypes, with ranges of 2.5 to 10.0% and 2.5 to 8.1% and averages of 5.8% and 4.8%, respectively. The TAG fraction ranged from 1.8% to 7.0% with an average of 3.3%. Figure 7.11 shows the proportions of each lipid fraction obtained from the 1D-TLC separation per genotype in ascending TFA content.

The FA compositions of each of these lipid fractions, averaged across genotypes, is given in Table 7.18. There was a slightly higher proportion of C16:0 in the FFA fraction compared to the other three lipid fractions. Polar lipids contained the largest proportion of C16:1*trans*-3 (1.9%) while FFA, TAG and DAG contained similar proportions of this FA (0.6%, 0.7% and 0.9%, respectively). Stearic acid (C18:0) was markedly higher in the TAG fraction (13.5%), FFA and DAG had similar proportions of this FA whereas POL had the least proportion (0.7%). The POL fraction also had the lowest proportion of C18:1*cis*-9 and C18:2n-6, which accounted for 1.6% and 9.4% respectively, while FFA, TAG and DAG had similar proportions of these FAs. However, POL contained the largest proportion of C18:3n-3 (72.4%). Free fatty acids (FFA) and DAG had 50.3% and 56.3% C18:3n-3 while TAG had the lowest proportion (33.8%). Also, TAG had the highest proportion of 'other' FAs, which was mainly influenced by the presence of C12:0, C20:0, C22:0 and C24:0.

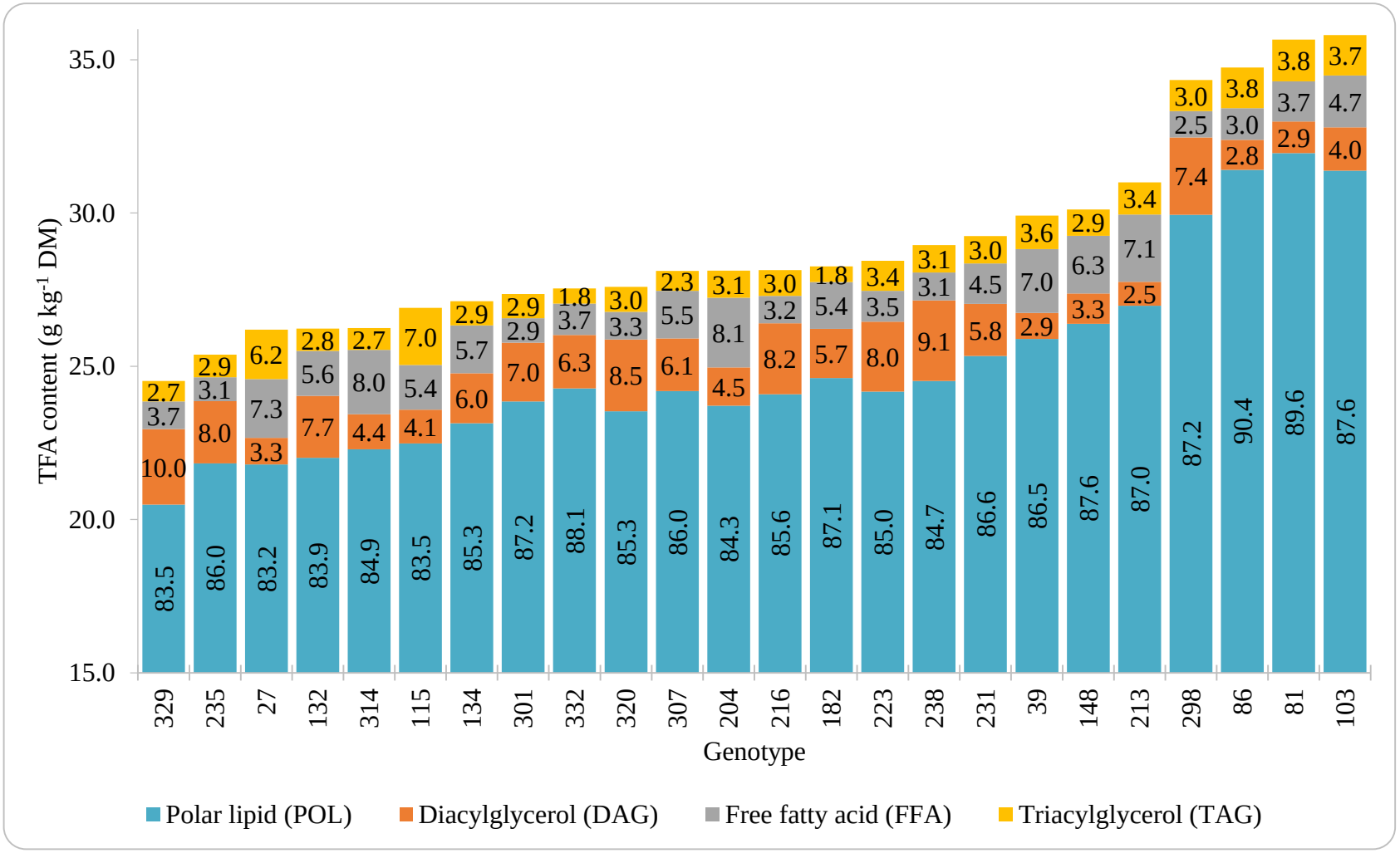

Figure 7.11 Proportions (%) of polar lipid (POL), diacylglycerol (DAG), free fatty acids (FFA) and triacylglycerol (TAG), resulting from 1D-TLC fractionation, per genotype in ascending total fatty acid (TFA) content (g kg^{-1} DM)

Table 7.18 Mean proportions of fatty acids (%) within each 1D-TLC lipid fraction

Lipid fraction	C16:0	C16:1 *trans*-3	C18:0	C18:1 *cis*-9	C18:2 n-6	C18:3 n-3	Other
POL	12.4	1.9	0.7	1.6	9.4	72.4	1.6
FFA	16.2	0.6	2.6	3.8	18.6	50.3	7.8
TAG	13.5	0.7	13.5	3.5	17.2	33.8	17.8
DAG	15.7	0.9	1.9	3.0	15.9	56.3	6.4

Abbreviations: POL, polar lipids; FFA, free fatty acids; TAG, triacylglycerol; DAG, diacylglycerol.

The relationships between total chlorophyll content, TFA content and the proportions of lipid fractions resulting for 1D-TLC separation are shown in Figure 7.12. Strong, positive correlations ($P<0.001$) were found between total chlorophyll content, TFA content and proportion of POL. The correlation between total chlorophyll and TFA was 0.8. The correlations of POL proportion with total chlorophyll and TFA were 0.85 and 0.71, respectively. All other correlations were on the whole low to negligible. Negative but negligible correlations were found between FFA proportion and total chlorophyll, TFA and POL proportion ($P>0.05$). No significant correlations were found with TAG proportion apart from TAG vs. DAG proportion ($r = -0.45$, $P<0.05$). However two of the samples, which had low total chlorophyll and TFA content, had distinctly higher TAG proportions compared to the other samples (>6% as opposed to the typical ~3%). Removal of these samples from the dataset revealed positive relationships for TAG proportion vs. total chlorophyll content ($r = 0.45$, $P<0.05$) and TAG proportion vs. TFA content ($r = 0.71$, $P<0.01$). Proportion of DAG was negatively correlated with all other variables ($P<0.05$).

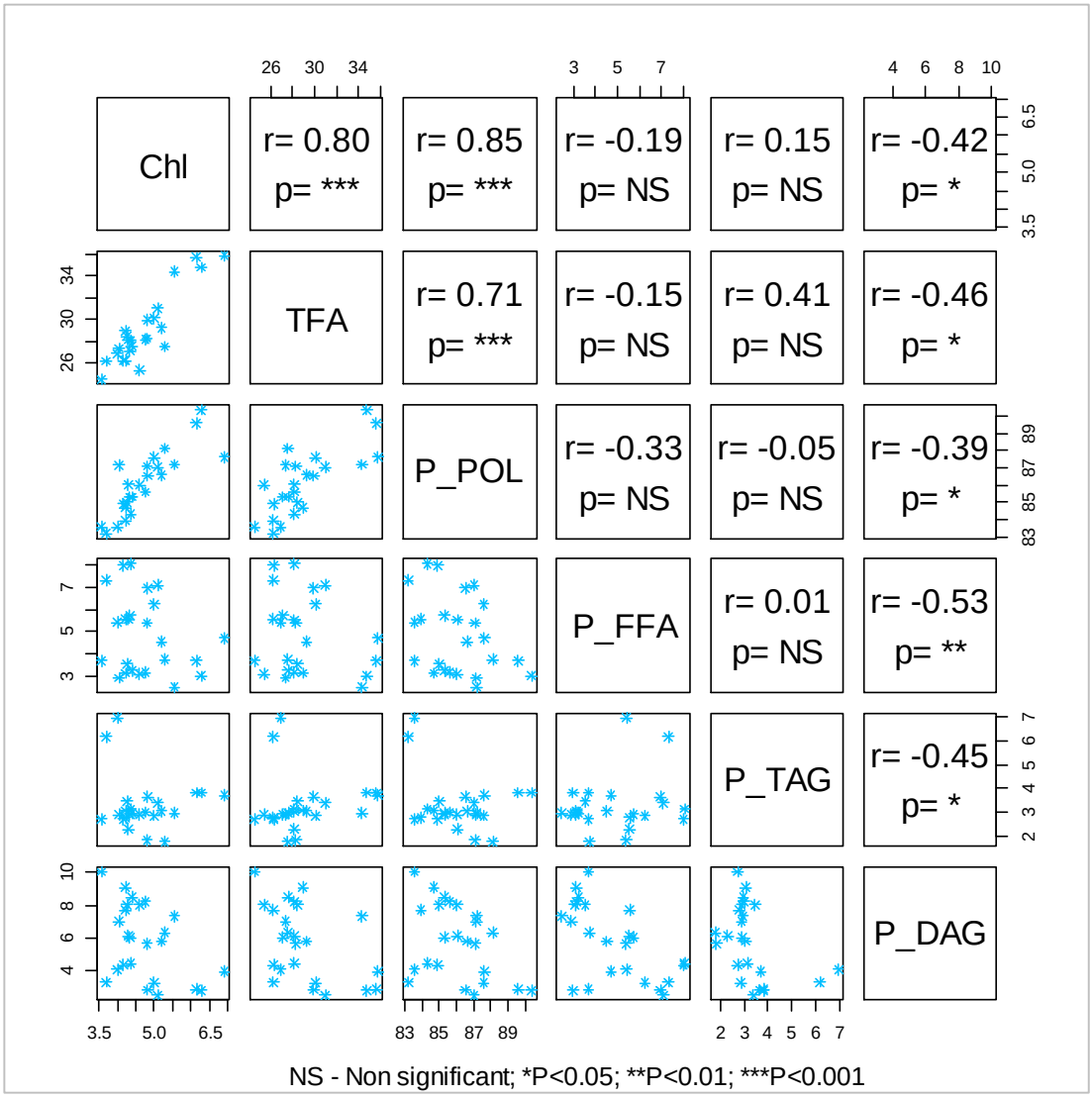

Figure 7.12 Scatter plot and Spearman's rank correlation matrix of total chlorophyll content, total fatty acid content and 1D-TLC lipid fractions.
Abbreviations: Chl, chlorophyll; TFA, total fatty acid; P_POL, proportion of polar lipid; P_FFA, proportion of free fatty acids; P_TAG, proportion of triacylglycerides; P_DAG, proportion of diacylglycerides.

7.4.2 SPE

The SPE method resulted in the separation of galactolipid (GL), phospholipid (PL) and neutral lipid (NL). Proportions of GL ranged from 65.4% to 76.9% with the across genotype average being 71.1%. Phospholipids (PL) accounted for the lowest lipid proportion, ranging from 10.6% to 13.7% with an average of 11.8%. Lastly, the average NL proportion was 17.0% and ranged from 12.3% to 21.2%. The proportions of each lipid fraction per genotype are shown in Figure 7.13.

The mean FA composition (%) of the SPE lipid fractions are presented in Table 7.19. Palmitic acid (C16:0) proportion was lowest in the GL fraction (9.4%), somewhat higher in the NL fraction (15.8%) and highest in the PL fraction (26.2%). Both GL and NL fractions had similarly low proportions of C16:1*trans*-3 whereas PL had a markedly higher proportion of this FA (6.9%). Galactolipid (GL) also had a low proportion of C18:0 (0.7%) while PL had a slightly higher proportion (1.4%) and NL had the highest proportion (7.9%). Similar proportions of C18:1*cis*-9 were found in PL and NL (3.1% and 3.4%, respectively) whereas GL had a lower proportion of this FA. Phospholipid (PL) and NL had somewhat similar C18:2n-6 proportions of 20.4% and 14.8%, respectively. In contrast, GL had a much lower proportion of C18:2n-6. The major FA in galactolipid was C18:3n-3, which accounted for 81.3%, whereas PL and GL had similar proportions of this FA (37.7% and 36.2%, respectively). A high proportion of 'other' FA was again found in the NL fraction, mainly due to the higher proportions of long-chain SFAs.

The relationships between total chlorophyll content, TFA content and proportions of lipid fractions from SPE separation are shown in Figure 7.14. Similar correlations were found between total chlorophyll content, TFA content and GL proportion

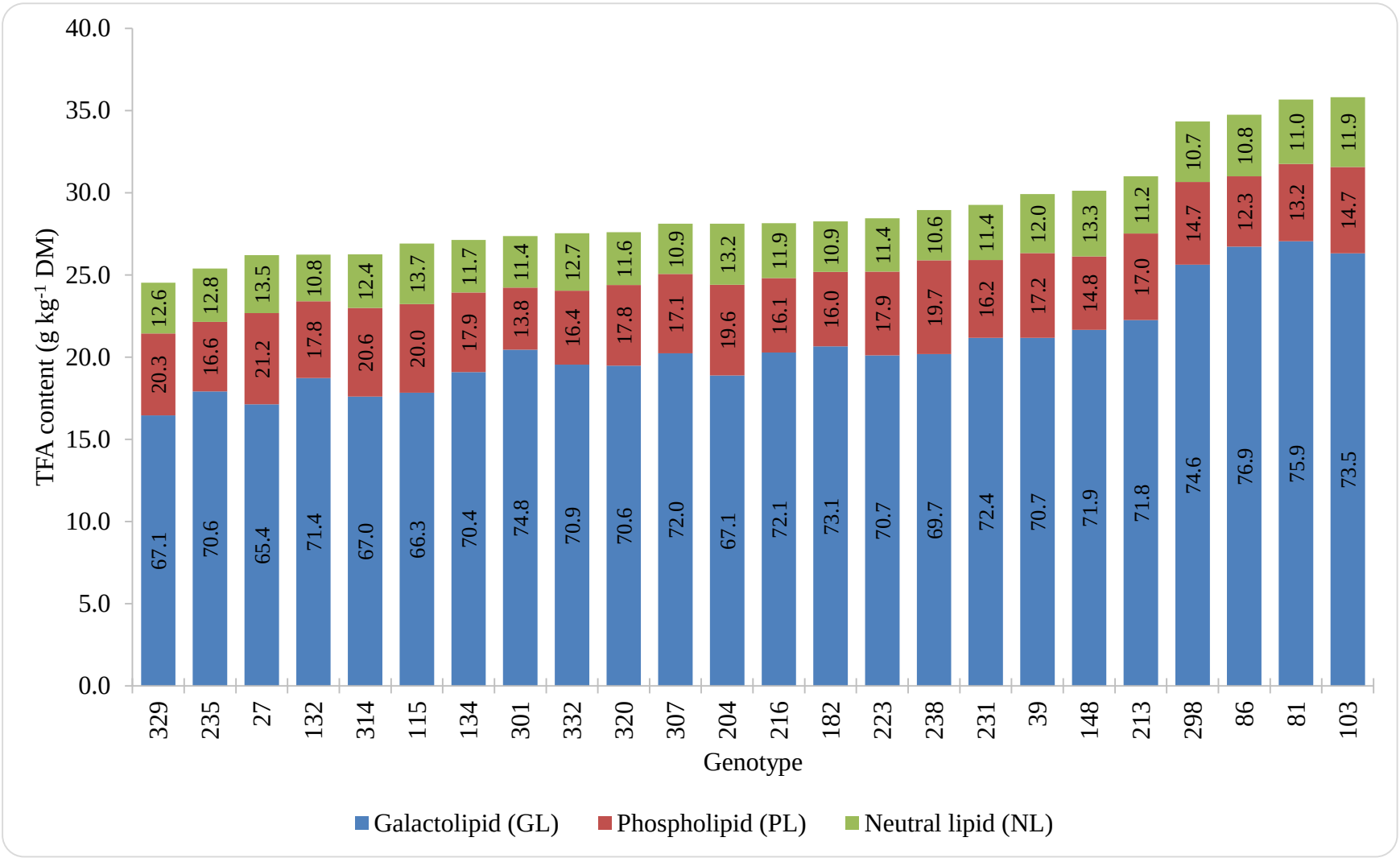

Figure 7.13 Proportions (%) of galactolipid (GL), phospholipid (PL) and neutral lipid (NL), resulting from solid phase extraction (SPE) fractionation, per genotype in ascending total fatty acid (TFA) content

Table 7.19 Mean proportions of fatty acids (%) within each SPE lipid fraction

Lipid Fraction	C16:0	C16:1 *trans*-3	C18:0	C18:1, *cis*-9	C18:2n-6	C18:3 n-3	Other
GL	9.4	0.7	0.7	1.0	4.1	81.3	2.8
PL	26.2	6.9	1.4	3.1	20.4	37.7	4.3
NL	15.8	0.4	7.9	3.4	14.8	36.2	21.4

Abbreviations: GL, galactolipid; PL, phospholipid; NL, neutral lipid.

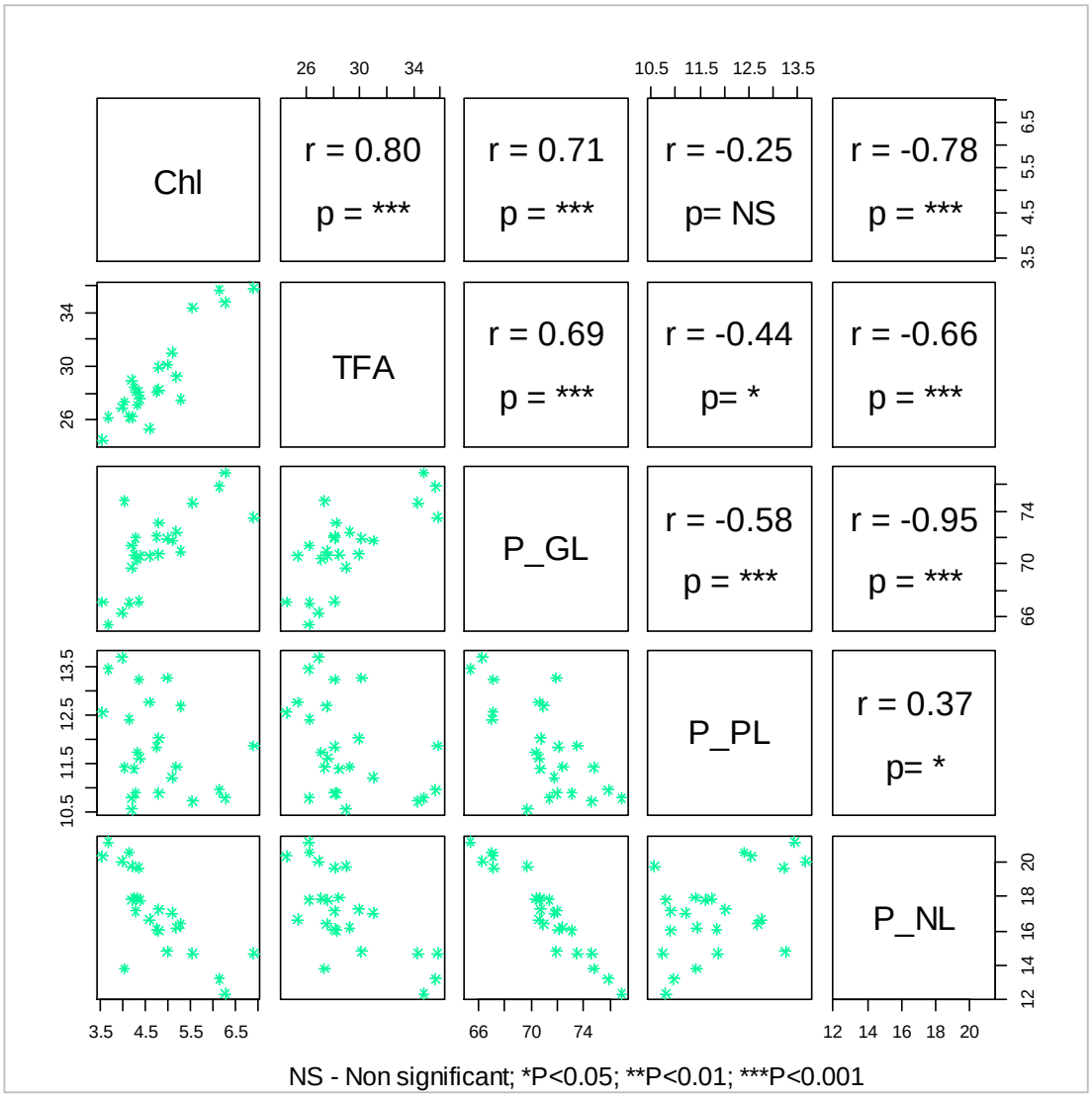

Figure 7.14 Scatter plot and Spearman's rank correlation matrix of total chlorophyll content, total fatty acid content and SPE lipid fractions.
Abbreviations: Chl, chlorophyll; TFA, total fatty acid; P_GL, proportion of galactolipid; P_PL, proportion of phospholipid, P_NL, proportion of neutral lipid.

(P<0.001). The correlation between total chlorophyll and TFA was 0.8, while total chlorophyll and TFA had almost identical correlations with GL proportion of 0.71 and 0.69, respectively. Neutral lipid (NL) proportion was negatively correlated with total chlorophyll, TFA and GL proportion (r = -0.78, -0.66 and -0.95, respectively; P<0.001). However the correlations with PL proportion were more variable, with low correlations for PL proportion vs. total chlorophyll (r = -0.25, P>0.05) and PL vs. NL proportion (r = 0.37, P<0.05). Moderate, negative correlations were found between PL proportion and TFA content (r = -0.44; P<0.05) and between PL and GL proportions (r = -0.58; P<0.001).

7.5 Discussion

7.5.1 Method Development and Limitations

The process of establishing and developing a laboratory method which produced the desired level of lipid separation to enable investigation of the original hypothesis was challenging. After exploration of the literature, 2D-TLC seemed to be the most popular means to achieve detailed separation of the galactolipids and phospholipids. However, large variation was found between the procedures used, especially in terms of the solvent eluents used and the methods of visualising and analysing the lipid fractions. Also, it seemed that no one method was obviously preferred over another, making the decision of which particular method to pursue more complicated. Following discussions with colleagues, the method of Christie (2003) was selected since this method used solvents common to the eluents described by other publications and were readily available in the laboratory. Also, a schematic of the resulting lipid separation of this method was available in the publication, which would assist with

evaluating the achieved lipid separation and with correct identification of the lipid fractions.

None of the publications gave an indication of the time required to run the respective methods. Thus, the first stage of method development involved using a 'test grass' lipid extract with the purpose of establishing and becoming familiar with what to expect from the method. From this first stage, it was established that from spotting the sample on the TLC plate to completing methylation would take the greater part of two-days, plus a further day before results from the GC-FID analysis of the FAMEs would become available. This meant that a maximum of two method runs could be achieved per week. However, with the need to interpret and discuss the outcomes of each method development stage with colleagues, this was reduced to approximately one method run per week.

Some good progress was made during the development of the 2D-TLC method, for example finding that the UV-A lamp gave improved and more defined fluorescence of the lipid fractions in contrast to the UV_{254} lamp. Nonetheless, a number of difficulties were also encountered. Many of these could be solved with continued minor adjustments of the technique however a key concern was poor recovery of the lipid from the adsorbent. After personal communication with Dr. Christie, it was decided to introduce an overnight methylation in place of the standard 2 hr methylation stated in the method of Sukhija and Palmquist (1988). The issue with recovering the lipids from the adsorbent may be due to the need for plates to be completely dried before visualising and removing the lipids. Although the overnight methylation did improve the recoveries, particularly of the phospholipids, the addition of this step to the already lengthy method would mean only one complete run would be achievable per week. In addition to this, there were still some minor issues surrounding the

optimal conditions for good separation of the lipids. Taking these unresolved issues into account, it was decided to try an alternative method.

Solid phase extraction (SPE) was chosen as this method does not require drying of the adsorbent, in fact this is discouraged during an SPE lipid separation; therefore it was believed that this method of lipid separation may give improved lipid recoveries. An adaption of the method of Yao and Rastetter (1985) was used which resulted in three lipid fractions: neutral lipids (NL), galactolipids (GL) and phospholipids (PL). Although the complexity of lipid separation and the amount of information generated from this method was much less compared to that from 2D-TLC, it was still sufficient to enable investigation of the original hypothesis that genotypes with higher TFA content would have higher chloroplast membranes (galactolipids), as these genotypes also had higher total chlorophyll content. The development of the SPE method was much more straightforward compared to the 2D-TLC method and yielded improved and more consistent lipid recoveries.

Using twenty-four composite samples from one harvest is one key limitation to this study in terms of experimental design. This was necessary due to the limited amount of plant material remaining from the field experiment described in Chapter 5. Cut 1 was the only cut where freeze-dried material was still available from all replicates of each genotype, however there was not enough material per replicate therefore bulk composite samples representing each genotype were obtained to ensure sufficient experimental plant material was available for lipid analysis.

7.5.2 Lipid Composition of Genotypes

The lipid proportions found in the present study are different to those reported by Chow *et al.* (2004) for fresh samples of three different perennial ryegrass cultivars (Barnhem, Agri and Respect). Averaged across cultivars, Chow *et al.* (2004) reported approximately 25% lower polar lipids, 7% higher TAG and more than 20% higher FFA compared to the present study. These compositional differences may be due to a number of factors such as cultivar differences, influence of season, effects of sample collection and handling procedures or differences in lipid separation technique. The samples used in the present study were harvested in early June after 29 days regrowth whereas the samples used in the study of Chow *et al.* (2004) were collected in August after 40 days regrowth. The higher proportions of FFA and TAG reported by Chow *et al.* (2004) may also be indicative of plant-mediated lipolysis in response to stress placed on the plant during harvesting and sample handling. This trend is especially evident in studies which have investigated the effects of drying, wilting and ensiling on lipid composition (Lee *et al.*, 2002, 2004; Fievez *et al.*, 2004; Van Ranst *et al.*, 2009). The mechanism behind increasing TAG during periods of stress is believed to be due to the upregulation of diacylglycerol acyltransferase, which converts DAGs released by the degradation of galactolipids into TAGs (Sakaki *et al.*, 1990). In the present study, individual plants were snap-frozen in liquid N within 10 min of harvesting, temporarily stored on dry-ice then stored at -20^{o}C until further analysis. In contrast, Chow *et al.* (2004) collected plant material from cultivar plots cut using a forage harvester within 20 min after cutting which were placed into sealed plastic bags then frozen within 15 min at -18^{o}C for storage. Nonetheless, Lee *et al.* (2003) reported the lipid composition of fresh samples of perennial ryegrass

being 73% POL, 13% DAG, 8% TAG and 7% FFA, which are more in-line with the results of the present study.

7.5.3 Fatty Acid Composition of Lipid Fractions

The POL fraction had higher proportions of C16:1*trans*-3 and C18:3n-3 compared to the other three lipid fractions of the 1D-TLC lipid separation. This fraction contains both galactolipids and phospholipids which typically have higher amounts of these FAs compared to the neutral lipids. Referring back to the lipid synthesis schematic in Chapter 2 (Figure 2.5), C16:1*trans*-3 is exclusively found in PG (Gao *et al.*, 2009), while C18:3n-3 is the predominant FA in found in the galactolipids (MGDG, DGDG and SQDG) but can also be found in smaller quantities in the phospholipids. The FA compositions of the FFA and DAG fractions were quite similar whereas TAG had lower proportion of C18:3n-3 and larger proportions of C18:0 and 'other' FAs. Lin and Oliver (2008) stated that the main FAs found in leaf TAG were C16:0, C18:0 and C18:1*cis*-9, however variation was observed between different plant species in terms of total amount and FA composition of TAG.

Hudson and Karis (1974) investigated the effect of crop maturity on lipid composition of fodder radish leaves. Table 7.20 presents their findings regarding the proportions of FAs (%) within lipid fractions at two different stages of maturity (28 and 56 days after sowing). Linolenic acid (C18:3n-3) was the chief FA in the galactolipid fractions (MGDG, DGDG and SQDG) and PE fraction, accounting for over 50% of the total FAs per lipid fraction. The present study found that C18:3n-3 was the predominant FA in all lipid fractions, including the neutral lipids (FFA, TAG and DAG from 1D-TLC, NL from SPE). Consequently, the neutral lipid fractions of

Table 7.20 Proportion of fatty acids (%) of the lipid classes of fodder radish at two different crop maturities (adapted from Hudson and Karis, 1974)

Lipid fraction	Crop maturity[1]	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	other
MGDG	*28*	4.8	-	2.6	35.0	1.9	54.0	1.7
	56	2.1	-	-	39.2	1.2	52.9	4.6
DGDG	*28*	19.6	2.7	7.3	2.7	6.5	57.7	3.5
	56	10.0	5.8	0.5	2.7	2.8	72.6	5.6
SQDG	*28*	17.7	1.1	0.9	14.7	9.5	51.3	2.8
	56	30.9	1.3	2.8	10.9	8.7	37.3	8.1
PE	*28*	14.1	1.1	0.5	17.9	7.5	52.5	6.4
	56	13.7	1.2	0.9	20.8	14.0	47.0	2.4
TG	*28*	-	-	-	-	-	-	-
	56	24.5	-	7.1	20.7	9.8	9.7	28.2
FA	*28*	-	-	-	-	-	-	-
	56	18.7	-	8.1	17.6	3.5	18.2	33.9
MG + DG	*28*	31.8	0.8	8.8	8.9	6.5	18.1	25.1
	56	27.2	-	12.6	7.8	6.2	20.1	26.1

[1]Days after sowing.
Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PE, phosphatidylethanolamine; TG, triacylglycerol; FA, fatty acid; MG + DG, partial glycerides.

the present study had lower proportions of C16:0 and C18:1*cis*-9 compared to those reported by Hudson and Karis (1974). These compositional differences may be due to the difference in plant species studied.

7.5.4 Relationships between Chlorophyll, Fatty Acids and Lipids

The positive correlation between chlorophyll content and TFA content reported here is keeping with the results previously reported by Mayland *et al* (1976) and Dierking *et al* (2010), in addition to the results presented in Chapter 5. The 1D-TLC lipid separation and subsequent statistical investigation into the relationships of these lipid fractions with chlorophyll and TFA content revealed positive correlations between polar lipid proportion with chlorophyll content and TFA content. This suggests that genotypes with higher TFA content also have higher proportions of polar lipids relative to the neutral lipids. Interestingly, the correlations between the neutral lipids (FFA, TAG and DAG) were overall quite low and variable, with no one type of neutral lipid being predominantly affected by increased polar lipid proportion.

Upon further separation of the polar lipids into galactolipids (GL) and phospholipids (PL) via SPE, it was discovered that GL was the chief lipid fraction contributing to FA content and was typically increased in the higher TFA genotypes; as indicated by the strong positive correlation between GL and TFA. An equally strong positive correlation was also found between GL and total chlorophyll. This triadic relationship can be attributed to the co-localisation of these elements within the thylakoid membranes of the chloroplast. As discussed previously, galactolipids form the major lipid component of chloroplasts, particularly in the thylakoid membranes of the chloroplast lamellae, which are 50% lipid by weight (Hawke, 1973). Of course, these galactolipids also contain FAs, particularly PUFA which play an important structural feature in photosynthesis and an essential role in the maintenance of the

electron transport system (Williams *et al.*, 1983; Routaboul *et al.*, 2000). Chlorophyll is also present in the thylakoid membranes, hence a strong relationship is observed between these three constituents.

The proportions of the PL and NL fractions were found to have negative correlations of varying strength with total chlorophyll, TFA and proportion of GL, yet a positive correlation was observed between these two lipid fractions. The proportion of NL in leaves is typically small, and the biological roles NLs play in leaves are still unresolved. One such role which has been put forward is that TAG is merely an intermediate of membrane degradation in response to stress or senescence (Sakaki *et al.*, 1990; Kaup *et al.*, 2002). Historically, it was a common assumption that leaf tissue did not contain any storage lipid (which is predominantly viewed to be TAG), especially in the form of oil bodies (sometimes referred to as spherosomes of oleosomes) which are found in seeds and fruits. These originate from the ER and consist of TAG droplets surrounded by a unique membrane comprising of a single layer of phospholipids and proteins known at oleosins (Taiz and Zeiger, 2007). However, some more recent work has provided evidence of the existence of oil bodies in the leaves of some plant species (Lersten *et al.*, 2006; Lin and Oliver, 2008). Indeed, Lin and Olive (2008) argue that the FA compositions of the TAGs found in their plant studies differed from the FA composition of the galactolipids. If it were the case that the TAGs found were due to stress or senescence mediated degradation and conversion of membrane lipid to TAG via diacylglycerol transferase, the FA composition of the TAGs and galactolipids would be similar. The FA compositions of the TAG / NL and the GL fractions examined in the present study were also different, thus potentially indicating that oil bodies exist in leaf tissue of perennial ryegrass. Furthermore, this could partly explain the positive relationship discovered between NL and PL fractions.

7.6 Conclusions

These results show that perennial ryegrass genotypes with increased TFA content have increased proportions of galactolipids, in addition to increased total chlorophyll content. However, further investigation of the lipid composition of perennial ryegrass, along with other forages, is warranted for a number of reasons. Firstly, these results require further validation using a larger sample size and material from a wider selection of cultivars/species. Secondly, to establish whether the increase in galactolipids is due to an increase in chloroplast size (due to increased amount of thylakoid membranes) or to an increase in the number of chloroplasts and the effects this may have on plant performance. Thirdly, to investigate environmental effects such as season on lipid composition of forages in order to enhance the understanding of FA acid and lipid metabolism in forages during a growing season. And lastly, to establish an understanding of the genetic control underpinning lipid composition of forages.

Chapter 8. Evaluation of NIR and FTMIR Spectroscopy to Predict Total and Individual Fatty Acid Content

8.1 Summary

This study evaluated the potential of two infra-red spectroscopy methods for predicting FA content of perennial ryegrass. Data generated from the study described in Chapter 4 involving one harvest of ninety-six plants (twenty-four genotypes with four replicates) maintained under polytunnel conditions was used to assess FA prediction by near-infrared reflectance (NIR) spectroscopy. The potential of Fourier-transform mid-infrared (FTMIR) spectroscopy to predict FA content was also assessed using plant material collected during the five harvests of the field study described in Chapter 5. Both NIR and FTMIR spectroscopy showed good potential in estimating total and individual FA content. The calibrations created in this study are sufficient for screening of plants for the majority of FAs except C18:1*cis*-9. With the addition of larger datasets consisting of a wider variety of plant material, more accurate calibrations could be achieved, which may lead to IR spectroscopy being used an alternative method to quantify FAs.

8.2 Introduction

The conventional wet chemistry techniques used to measure plant chemical composition are typically time-consuming, destructive and expensive, may involve hazardous chemicals and require a skilled analytical technician (Foley *et al.*, 1998; Foster *et al.*, 2006). Near-infrared reflectance (NIR) spectroscopy offers a much more rapid, non-destructive and relatively low cost alternative which, with proper

development and maintenance of calibrations, can give accurate estimations of feed composition (Corson *et al.*, 1999). NIR spectroscopy is also capable of analysing multiple constituents in a single operation with minimal sample preparation (Marten *et al.*, 1989). As a result, it has been widely adopted within agricultural, food and manufacturing industries for compositional and functional analysis of products. One of the major applications of NIR spectroscopy within agriculture has been in the evaluation and improvement of the nutritional value of forage (Norris *et al.*, 1976; Abrams *et al.*, 1987; 1989; Baker and Barnes, 1990; Shenk and Westerhaus, 1994). Characteristics such as moisture content, CP, WSC, fibre (ADF, NDF and lignin) and IVOMD are now routinely measured in this way.

The procedure relies on molecular overtone and combination vibrations seen in the near-infrared spectral region (730 - 2,500 nm) by C-H, N-H and O-H bonds, which are the primary constituents of the majority of organic compounds found in plants and animals (Osborne, 1993; Givens *et al.*, 1997; Foley *et al.*, 1998). When near-infrared radiation is passed through a dried, milled sample (transmission and diffuse reflectance), the light can interact with the sample in a number of ways. Either it shines onto the sample and is reflected straight back, or more often it interacts with the sample before being reflected back towards the detector. It is the spectrum of this reflected light that contains the chemical composition details of the sample (Shenk and Westerhaus, 1994; Givens *et al.*, 1997). Due to the vibration bands typically being broad and overlapping, this leads to a complex spectra making it difficult to assign specific features of the spectrum to specific chemical components. However, this is overcome by use of multivariate techniques to extract the desired chemical information.

Fourier transform mid-infrared (FTMIR) spectroscopy also works on similar principles; except it uses a different region of the electromagnetic spectrum (400 - 4,000 cm^{-1}) and also involves 'Fourier transform' which is the conversion of light absorption per mirror position to light absorption per wavelength. Additionally, it is based on fundamental molecular vibration bands, rather than overtones and combinations of these bands, making the spectrum comparatively less complex than NIR and also making it possible to assign certain regions of the spectrum to certain chemical components. Although FTMIR is much less appraised in terms of commercial forage analysis, it is becoming more frequently used within research environments. In terms of plant composition, Allison *et al.* (2009a, 2009b) have demonstrated the capability of FTMIR to predict cell wall components (such as lignin), C and N content of energy grasses. More recently, Belanche *et al.* (2013) reported comparable FTMIR prediction accuracies to those of NIR when predicting the CP content of a range of forages. The advantages of FTMIR over NIR spectroscopy is that it is capable of superior resolution and often scans a much smaller sample size (Griffiths, 1983; Laurens and Wolfrum, 2011).

Once adequate spectroscopic and reference method data have been collected, calibration equations are then developed using multivariate statistical methods, such as modified partial least squares (PLS) regression, which relate the infrared spectral data with the reference data (Windham *et al.*, 1989). The quality of calibration equations is commonly assessed by means of linearity and accuracy. Generally, linearity is assessed via the coefficient of determination (R^2), with higher R^2 values indicating better linearity, while accuracy is assessed using standard error values (Landau *et al.*, 2006). Optimum calibration equations are usually those with the highest R^2 value and lowest standard error values. There are several types of standard error

related to calibration development. Some minor variability exists in the specific terminology used to define these standard errors, however they can be broadly split into three types: the standard error of calibration (SEC), standard error of prediction (SEP) and standard error of cross-validation (SECV). The SEC is a measure of the variability in the difference between predicted vs. observed values for the data used for developing the calibration, referred to as the 'calibration' or 'training' dataset. The SEP on the other hand is a measure of the variability in the difference between predicted vs. observed values when the equation is applied to an 'independent' or 'test' dataset used to validate the calibration. However, this method of validation has been superseded by cross-validation, which involves sequentially applying the calibration equation to subsets of the calibration dataset (Foley *et al.*, 1998). This results in a SECV which represents the variability in the difference between predicted vs. observed values of the cross-validated data. Another statistic which has historically been used to assess the performance of a calibration model was the ratio of performance to deviation (RPD). This is the ratio of the SEP of the calibration model to the standard deviation (SD) of the reference data. However, Minasny and McBratney (2013) recently demonstrated that RPD and R^2 are inadvertently the same measure.

The aim of this piece of work was to establish calibration equations using previous FA data coupled with NIR and FTIR spectroscopic data to predict individual and total FA content of perennial ryegrass. The accuracy, performance and practicality of these calibrations would then be assessed using R^2 and standard error values.

8.3 Materials and Methods

8.3.1 Fatty Acid Determination

Fatty acid analysis was carried out as described in Chapter 3, using the methylation procedure of Sukhija and Palmquist (1988) and quantification via GC-FID.

8.3.2 NIR Spectroscopic Analysis

8.3.2.1 Plants

Fatty acid and NIR spectroscopy data generated from the experiment described in Chapter 4 was used to establish calibration models between FAs and NIR. The experiment involved four genotypes from the Aurora x AberMagic F1 mapping population and twenty genotypes from the B674G breeding population, with four replicates of each genotype giving a total of ninety-six plants. These plants were maintained under poly-tunnel conditions with one harvest collected in July 2012 for FA and NIR spectroscopy analysis.

8.3.2.2 NIR Spectroscopy

Approximately 2-5g of freeze-dried sample was packed into red cells and each scanned once at 2 nm intervals over the wavelength range from 400 to 2500 nm in reflectance mode, using a scanning monochromator (FOSS NIRSystems 6500, FOSS UK Ltd., Warrington, UK). Data were collected using WinISI II software (Version 1.02a, FOSS, Infrasoft International, Port Matilda, USA) and spectra were stored as log 1/R where R is the diffuse reflectance. Data over wavelength range 1100 to 2498 nm were used to develop calibrations for FAs using WinISI 4 (Version 4.6.8, FOSS Analytical A/S).

8.3.2.3 Calibration Model

NIRS spectra were subjected to standard normal variate (SNV) and detrend (DT) scatter corrections along with first (1,4,4,1) and second (2,6,4,1) derivative math treatments. Modified PLS regression was carried out using WinISI 4 (Version 4.6.8, FOSS Analytical A/S, Slangerupgade 69, 3400 Hilleroed, Denmark) with group cross-validation. Outliers were identified as samples with a critical t-statistic of >2.5 or global-H value of >10 and were removed from the dataset. Optimal math treatments and calibrations were selected based on a minimum standard error of cross-validation (SECV) and maximum coefficient of determination (R^2). Calibration equations were calculated for C16:0, C16:1*trans*-3, C18:0, C18:1*cis*-9, C18:2n-6, C18:3n-3 and TFA.

8.3.3 FTMIR Spectroscopic Analysis

8.3.3.1 Plants

The FA data presented in Chapter 5 was paired with FTMIR spectroscopy data to establish calibration models between FAs and FTMIR spectroscopy. The experiment presented in Chapter 5 involved the same ninety-six plants described earlier in this chapter maintained under field conditions. These were subjected to a simulated grazing management regime which began in May 2013. A total of five harvests were collected and analysed for FA content: Cut 1 (5th Jun 2013), Cut 2 (1st Jul 2013), Cut 3 (1st Aug 2013), Cut 4 (28th Aug 2013) and Cut 5 (24th Sep 2013).

8.3.3.2 FTMIR Spectroscopy

Freeze-dried plant material was analysed using an Equinox 55 FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) fitted with a Golden Gate attenuated total reflectance (ATR) accessory (Specac Ltd., Slough, UK). Samples were analysed in triplicate from 700 to 4500 cm^{-1} in 2 cm^{-1} intervals and corrected for background

absorbance by subtraction of the spectrum of the empty ATR crystal. Absorbance spectra were collected and converted to text files in Opus software (version 4.2, Bruker UK Ltd., Coventry, UK) for subsequent data analysis.

8.3.3.3 Calibration Model

Fatty acid and FTMIR spectroscopy data was available for a total of 467 samples. Replicate spectra were averaged to give a single spectrum per sample using Excel 2013 (Microsoft, Redmond, Washington State, USA). Spectra were then mean-centre (vector) normalised using MatLab 2013a (MathWorks, Natick, Massachusetts, USA). Data were randomised and divided into two-thirds training data (n = 313) and one third test data (n = 154). Modified PLS regression with 10-fold cross-validation was carried out on the training dataset using MatLab 2013a, to generate a calibration equation between FTMIR and FA data. The number of components used in the final model were selected based on when the mean squared error of cross-validation (MSECV) reached a minimum. The test dataset was then used to test the model independently. Coefficient of determination (R^2), MSECV and mean square error of prediction (MSEP) values were used to evaluate the predicted vs. observed values for both training and test datasets. Calibration equations were calculated for C16:0, C16:1*trans*-3, C18:0, C18:1*cis*-9, C18:2n-6, C18:3n-3 and TFA.

8.4 Results

8.4.1 NIR Spectroscopy Calibration Model

Scatter corrected spectra for all 96 samples scanned using NIR spectroscopy are shown in Figure 8.1. A summary of the FA data and optimum calibration results for individual and total FAs is given in Table 8.1. Any outlying data points were omitted during calibration development, resulting in between 89 and 95 samples being used for calibration of FAs with NIRS. Second derivative math treatments performed better for prediction of C16:0, C16:1*trans*-3, C18:2n-6, C18:3n-3 and TFA while first derivative math treatments were superior for prediction of C18:0 and C18:1*cis*-9.

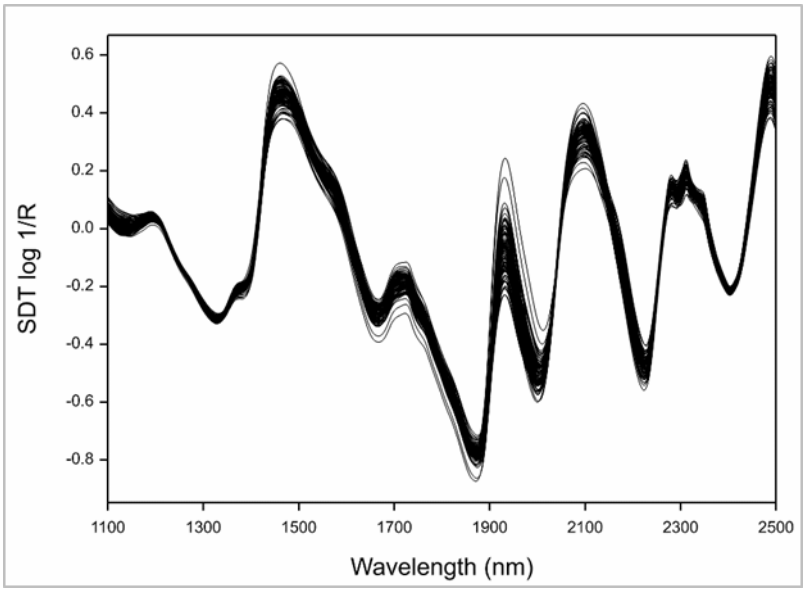

Figure 8.1 Scatter corrected (SDT) NIRS spectra for all 96 samples scanned from 1100 to 2500nm

A reasonable amount of variation was present in the FA data used for calibration development, with coefficients of variation (CV%) ranging from 14.2% to 19.9% for C16:0, C18:0, C18:1*cis*-9, C18:2n-6 and TFA. Higher variation was present for C16:1*trans*-3 and C18:3n-3 content, with CV% of 31.9% and 26.8% respectively. The

Table 8.1 Calibration statistics for prediction of individual and total fatty acid content of perennial ryegrass using NIR spectroscopy

Fatty Acid	Math treatment	*n*	Min	Max	Mean (g kg^{-1} DM)	SD	CV%	No. of Terms	SEC	R^2	SECV	1-VR
C16:0	2,6,4,1	92	2.64	5.21	3.93	0.560	14.2	5	0.160	0.92	0.189	0.88
C16:1*trans*-3	2,6,4,1	94	0.13	0.71	0.37	0.118	31.9	7	0.030	0.94	0.037	0.90
C18:0	1,4,4,1	89	0.28	0.49	0.37	0.056	15.1	7	0.025	0.80	0.030	0.71
C18:1*cis*-9	1,4,4,1	95	0.39	0.78	0.58	0.084	14.5	6	0.058	0.53	0.071	0.29
C18:2n-6	2,6,4,1	94	2.48	4.92	3.64	0.549	15.1	6	0.193	0.88	0.247	0.76
C18:3n-3	2,6,4,1	92	6.28	20.67	12.74	3.411	26.8	6	0.711	0.96	0.853	0.94
Total	2,6,4,1	92	14.48	33.79	23.30	4.637	19.9	6	0.950	0.96	1.095	0.94

SD, standard deviation; CV%, coefficient of variation; SEC, standard error of calibration; R^2, coefficient of determination for calibration; SECV, standard error of cross-validation; 1-VR, coefficient of determination for cross-validation (one minus the ratio of unexplained vs. total variance).

ranges in content for C16:1*trans*-3 and C18:0 were very similar (0.13 to 0.71 and 0.28 to 0.49 g kg^{-1} DM, respectively) with both averaging at 0.37 g kg^{-1} DM. These FAs also had similar SECV values of 0.037 and 0.030 respectively. Oleic acid (C18:1*cis*-9) content was slightly higher, ranging from 0.39 to 0.78 g kg^{-1} DM, averaging at 0.58 g kg^{-1} DM with a SECV of 0.071. Palmitic acid (C16:0) and C18:2n-6 were also similar in range, mean and SECV while C18:3n-3 ranged from 6.28 to 20.67 g kg^{-1} DM, with an average of 12.74 g kg^{-1} DM and a SECV of 0.853. Total FA ranged from 14.48 to 33.79 g kg^{-1} DM and averaged at 23.30 g kg^{-1} DM with a SECV of 1.095. The majority of calibrations had high R^2 values of >0.8, with very high R^2 values of 0.96 found for both C18:3n-3 and TFA, The exception to this was C18:1*cis*-9 which had a moderate R^2 of 0.53. Likewise, the 1-VR values followed a similar pattern, with the majority of calibrations having 1-VR values of >0.71, apart from C18:1*cis*-9 which had a low 1-VR value of 0.29.

8.4.2 FTMIR Spectroscopy Calibration Model

The mean-centred spectra for all 467 samples scanned via FTMIR spectroscopy is shown in Figure 8.2. A summary of the training and test FA data, along with calibration results is given in Table 8.2. Although a larger dataset was used for FTMIR calibration, a similar degree of variation was present in this data to that found in the smaller FA dataset used for NIRS calibration, exemplified by the CV% values. Furthermore, the range, SD and CV% of constituent and total FAs were also very similar between the training and test datasets, and thus datasets were appropriate for calibration development and independent testing.

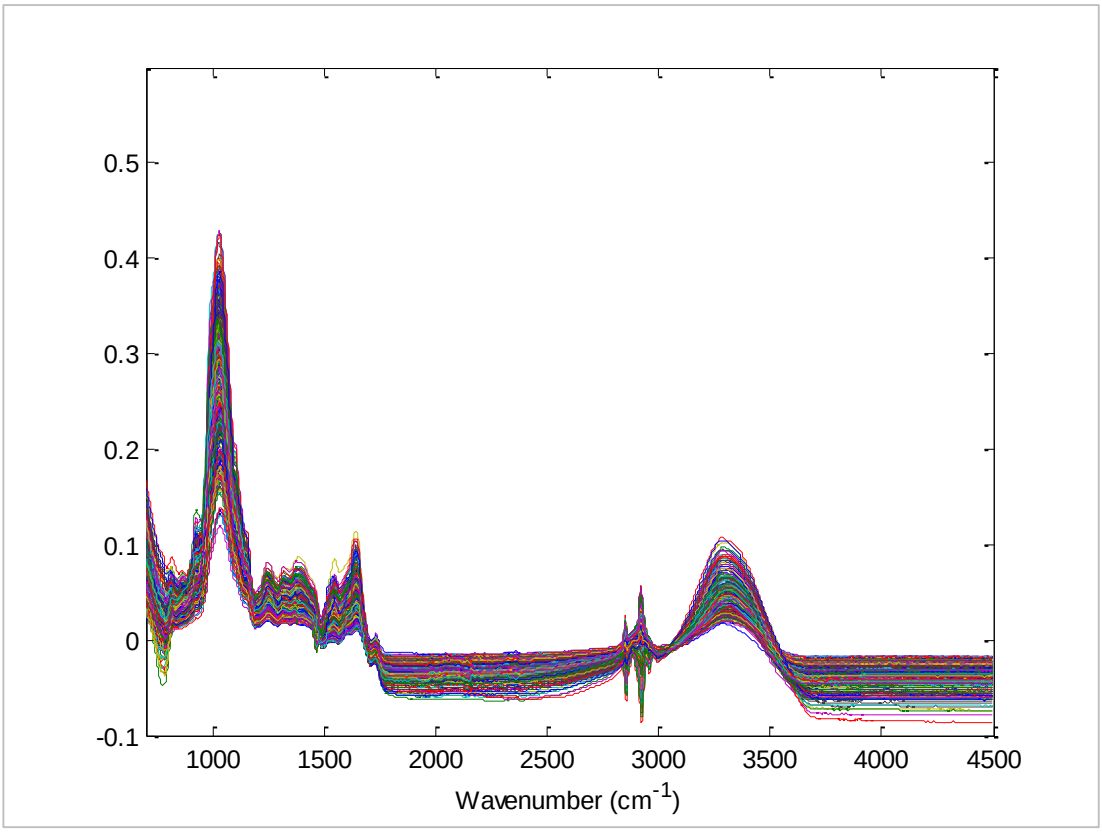

Figure 8.2 Mean-centred FTMIR spectra of all 467 samples scanned from 700 to 4500cm^{-1}

In terms of the training dataset, the ranges in content of C16:1*trans*-3, C18:0 and C18:1*cis*-9 were 0.23 to 1.24, 0.24 to 0.81 and 0.34 to 1.03 g kg^{-1} DM. These FAs also had similar mean content of 0.57, 0.44 and 0.58 g kg^{-1} DM and MSECV values of 0.007, 0.003 and 0.008, respectively. The range in C16:0 content was 3.11 to 6.63 g kg^{-1} DM while C18:2n-6 ranged from 2.44 to 6.04 g kg^{-1} DM. However both had virtually identical mean content (0.72 and 0.71 g kg^{-1} DM) and MSECV values (0.153 and 0.155). Alpha-linolenic acid (C18:3n-3) content ranged from 12.12 to 36.22 g kg^{-1} DM with an average of 21.26 g kg^{-1} DM and a MSECV of 5.759. Total FA ranged from 22.30 to 52.95 g kg^{-1} DM with an average of 33.58 g kg^{-1} DM and a MSECV of 9.585. High R^2 values were found for C16:0, C16:1*trans*-3, C18:0,

Table 8.2 Calibration statistics for prediction of individual and total fatty acid content of perennial ryegrass using FTMIR

	Training (n = 313)									*Test (n = 154)*						
Fatty Acid	**Min**	**Max**	**Mean**	**SD**	**CV%**	**No. of Components**	**MSEC**	**MSECV**	**R^2**	**Min**	**Max**	**Mean**	**SD**	**CV%**	**MSEP**	**R^2**
		(g kg^{-1} DM)									**(g kg^{-1} DM)**					
C16:0	3.11	6.63	4.67	0.72	15.4	10	0.128	0.153	0.75	2.71	6.66	4.74	0.83	17.5	0.130	0.81
C16:1*trans*-3	0.23	1.24	0.57	0.17	29.8	18	0.004	0.007	0.87	0.33	1.04	0.58	0.16	27.6	0.006	0.77
C18:0	0.24	0.81	0.44	0.10	22.7	13	0.002	0.003	0.77	0.22	0.71	0.44	0.10	22.7	0.003	0.70
C18:1*cis*-9	0.34	1.03	0.58	0.10	17.2	15	0.005	0.008	0.51	0.35	0.99	0.57	0.10	17.5	0.008	0.29
C18:2n-6	2.44	6.04	3.95	0.71	18.0	15	0.099	0.155	0.80	2.11	5.52	3.93	0.74	18.8	0.148	0.73
C18:3n-3	12.12	36.22	21.26	4.82	22.7	16	3.397	5.759	0.85	13.27	35.70	21.71	5.03	23.2	5.005	0.80
Total	22.30	52.95	33.58	6.31	18.8	9	8.295	9.585	0.79	21.88	51.54	34.09	6.75	19.8	8.305	0.82

SD, standard deviation; CV%, coefficient of variation; MSEC, mean square error of calibration; MSECV, mean square error of cross-validation; R^2, coefficient of determination; MSEP, mean square error of prediction.

C18:1*cis*-9, C18:2n-6, C18:3n-3 and TFA, ranging from 0.75 to 0.87. However, although C18:1*cis*-9 had a relatively low MSECV, a poorer R^2 value of 0.53 was found for this calibration.

As previously mentioned, the dataset used independently to test these calibrations had very similar range and variation to the training dataset. From this independent testing, the calculated MSEP values were marginally lower but very similar to the MSECV values calculated during calibration development. The R^2 values for predicted vs. observed of the test dataset were slightly higher for C16:0 and TFA (0.81 and 0.82, respectively), slightly lower for C16:1*trans*-3, C18:0, C18:2n-6 and C18:3n-3 (0.77, 0.70. 0.73 and 0.80, respectively) and markedly lower for C18:1*cis*-9 (0.29).

8.5 Discussion

Detailed discussion of the FA data used for NIR and FTMIR calibrations can be found in Chapter 4 and Chapter 5, respectively. Successful calibrations were established by both spectroscopic methods for C16:0, C16:1*trans*-3, C18:0, C18:1*cis*-9, C18:2n-6, C18:3n-3 and TFA content. The NIR calibration data had good linearity with high R^2 values of >0.8, except for C18:1*cis*-9 which had an R^2 value of only 0.53. Although the FTMIR data set was much larger (467 samples) compared to the NIR data set (96 samples), the R^2 values for the FTMIR calibrations were slightly lower than those found for NIR. Nonetheless, they were still on the whole high (>0.7) with the exception of C18:1*cis*-9, which had respective training and test dataset R^2 values of 0.51 and 0.29. The reason for slightly lower linearity with the FTMIR calibrations may be due to the samples being derived from a number of harvests across a growing season, thus causing wider variability in the data compared to the NIR data set which was carried out on plants from a single harvest.

Foster *et al* (2006) and Dierking *et al* (2010) have previously investigated the capability of using NIR spectroscopy to predict FA content using data from a multitude of forage species maintained under glasshouse conditions. Foster *et al* (2006) reported calibrations for Lauric acid (C12:0), Myristic acid (C14:0), C16:0, Palmitoleic acid (C16:1*cis*-9), C18:0, C18:1*cis*-9, C18:2n-6 and C18:3n-3. Mean FA content was slightly higher in their study, which is probably due to the plants being maintained under different conditions compared to the present study (glasshouse vs. poly-tunnel). Also, the SD values of the data were slightly higher due to the inclusion of a range of grass, legume and forb species, thus creating wider variability in the dataset. As a result, SEC and SECV values were marginally higher but comparable to those found in the present study and all calibrations had higher R^2 (>0.93) and 1-VR values (>0.89) compared to the present study. Dierking *et al* (2010) also developed calibrations for these FA, plus myristoleic acid (C14:1); although their calibrations for C14:0 and C16:1 were unsuccessful. Again, mean, SEC and SECV values were marginally higher but comparable to the present study. They also calibrated NIR spectroscopy with total chlorophyll, chlorophyll *a* and chlorophyll *b*. The majority of the calibrations had very high R^2 values of >0.91, apart from C12:0 and C18:1*cis*-9 which had respective R^2 values of 0.83 and 0.72. A lower R^2 value was also observed in the present study for C18:1*cis*-9 with both NIR and FTMIR spectroscopy. Reasons for this are currently unclear, however it is unlikely to be related to the concentration of this FA as C16:1*trans*-3 is also present in similar quantities but has higher R^2 values (>0.77).

The study by Calderon *et al* (2007) evaluated and compared the performance of FTNIR and FTMIR in addition to NIR spectroscopy in predicting forage FA content, again using data from a variety of forage species maintained under glasshouse conditions. They reported calibrations for C12:0, C14:0, C14:1, C16:0, C16:1, C18:0,

C18:1*cis*-9, C18:2n-6 and C18:3n-3 and found similar R^2 and standard error values to those reported by Foster *et al* (2006). Some minor differences were observed between the types of spectroscopy used, but these differences were often small and all three spectroscopic methods performed satisfactorily. They did note that NIR had slightly better performance overall which was probably due to the instrument scanning a larger sample area. This trend was also observed in the present study with NIR calibrations having higher R^2 values than the FTMIR calibrations. However, the SEC and SECV values for the majority of the FAs were marginally lower for FTMIR, with the exception of C18:3n-3 and TFA which had markedly higher SEC and SECV values compared to the NIR calibrations.

The results of the present study, along with the previously published work by Foster *et al* (2006), Calderon *et al* (2007) and Dierking *et al* (2010), show that IR spectroscopy is a suitable tool for estimating the FA content of forage. Although 'narrow' single-species calibrations are often, but not always, more accurate, 'wide' multi-species calibrations have better practical application as they allow for the analysis of a wider selection of forages and mixed-sward pastures (Shenk and Westerhaus, 1993; Landau *et al.*, 2006). Therefore, Foster *et al* (2006) suggested inclusion of data from plants grown under a range of environmental conditions, including glass-house, poly-tunnel and field, involving a wide range of species at different developmental stages along with different sample preparation techniques (i.e. oven vs. freeze-dried). Furthermore, the recent development of hand-held 'on-line' NIR spectrometers is making the prospect of 'real-time' analysis of forage more plausible (Foley *et al.*, 1998). Thus, with appropriate calibration development, traditional forage quality parameters such as CP, WSC, fibre and digestibility, as well

as more novel traits such as FA content, could be monitored via IR spectroscopy within a breeding programme and be assessed instantaneously in the field (Foster *et al.*, 2006).

8.6 Conclusions

These results demonstrate that both NIR and FTMIR spectroscopy are capable of predicting TFA content of perennial ryegrass with comparatively high accuracy. Both methods can also predict the content of individual FAs with good accuracy, apart from C18:1*cis*-9. The accuracies of the present calibration models would suffice in screening for plants with relatively higher or lower FA content. However, addition of more data from a wider variety of forages cultivated under a range of conditions would aid to increase accuracies and potentially make quantification of FAs possible through the use of NIR or FTMIR spectroscopy. Additionally, FTMIR spectroscopy may have advantages early on in a breeding programme due to this method only requiring small amounts of sample. On the other hand, the larger sample size required for NIR spectroscopy may have advantages over FTMIR, particularly in terms of complex materials such as forages where a larger sample would be more representative, and thus may be better suited during plot-scale trials for example.

Chapter 9. General Discussion

9.1 Overview

This thesis set out to investigate the variation in and relationships between FAs, lipids and other characteristics of perennial ryegrass, along with some prospective methods to predict FA content. The key objectives were to (a) assess the genotypic and seasonal variation in FA and lipid content and composition; (b) investigate the effect variation in FA content has on other nutritional characteristics; (c) quantify the relationships between FA, lipids and other characteristics of perennial ryegrass; and (d) evaluate alternative methods to predict FA content of perennial ryegrass.

9.2 Study Limitations

The majority of the studies discussed in this thesis were carried out using a set of 96 plants (24 genotypes x 4 replicates), which comprised of four genotypes from an Aurora x AberMagic F1 mapping population and twenty genotypes from the B674G intermediate heading 13th generation breeding population. This is a comparatively small population for investigating a new trait, considering that plant trials are often quite extensive with the purpose of investigating hundreds of genotypes at a time. The genotypes used in the present studies were originally selected based on historic *SPAD*-502 measurements, due to the discovery of the positive relationship between *SPAD* and TFA, as described in Chapter 3. Genotypes were selected in this way with the aim of creating a sub-sample of genotypes which were representative of the overall variation in FA content of each population. This was achieved to some extent, as significant differences in terms of FA content and composition were found between

the genotypes. Although, they did not match up to their respective low through to high *SPAD* groupings for FA content.

The work discussed in Chapter 4 investigated the effects variation in FA content had on CP and WSC. A limitation of this study was the fact that only one harvest was taken from the plants while they were maintained in a poly-tunnel. This means that the results presented in Chapter 4 are only a snapshot of the relationships between FA, CP and WSC at the time of harvest. Ideally, several harvests would have been collected to investigate the effect of different stages of maturity or season.

The main experiment of this thesis is described in Chapter 5, which investigated the seasonal effects on chlorophyll content (*in vivo* and *in vitro*), FA content and composition as well as the relationships between these two characteristics. The main limitation of that experiment is that it was only carried out over one growing season. In order to confirm the seasonal effect observed, this work would require repetition over a number of years. However this was not possible due to the time constraints of this research programme. Also, analysis of these plants was carried out on leaf material only. Previous studies which have investigated FAs in forages have included the stems, with a limited number also calculating the leaf to stem ratio, which is known to influence FA content. The decision to only use the leaf material was so that inclusion of the stems did not interfere with establishing the inter-relationships between *in vivo* chlorophyll, *in vitro* chlorophyll and FAs as *in vivo* chlorophyll could only be measured on leaves. If stems were included in the sampled material from the plants, this would have a dilution effect on the *in vitro* chlorophyll and FA results.

Chlorophyll has good potential to be used as a proxy for TFA content of fresh forage, due to the consistently positive relationships reported across a growing season

in Chapter 5, in addition to earlier work that has investigated this relationship. With the development of tools such as hand-held chlorophyll meters and remote-sensing technologies, it is now possible to gain real-time estimates of chlorophyll content of plants and pastures in a non-destructive manner. The specific *in vivo* chlorophyll estimation tool used in the present studies was the *SPAD*-502 chlorophyll meter. Although a positive relationship was found between *SPAD* and TFA content in the study discussed in Chapter 3, this relationship was rather weak. Upon further investigation into the accuracy of *SPAD* in predicting chlorophyll content, it was found that the correlations between *in vivo* chlorophyll vs. *in vitro* chlorophyll were low to moderate and rather variable between cuts (discussed in Chapter 5). The *SPAD*-502 meter was developed by Minolta (Minolta corporation, Ltd., Osaka, Japan) to aid management of the nitrogen status of crops, and has been extensively used with rice, wheat and maize for example. However, the use of *SPAD*-502 with forages is much less common, which may be due to the design of the device not being suited to narrow-leaved plants. The *SPAD*-502 works by measuring the transmission of light through a 2 x 3mm rectangle of leaf area. This small area is sufficient when using the chlorophyll meter with broad-leaved crops such as rice, wheat or maize; however some difficulties arise when using it with narrow-leaved plants such as perennial ryegrass. For example, to gain a correct reading from the chlorophyll meter, practically the whole width of the leaf is used in order to cover the 2 x 3mm window where the transmission of light through the leaf is measured. The vast majority of the time, this means that the mid-rib section is also included in this 2 x 3mm area, which may interfere with the accuracy of the results as this part of the leaf is usually slightly thicker than the leaf blade. Either development of a chlorophyll meter which is suitable for use with narrow-leaved forage species or investigation of other methods of *in vivo* chlorophyll estimation is

needed in order to develop an instantaneous, non-destructive method of screening plants for high or low FA content. Chlorophyll estimation may also be a useful tool in monitoring plant FA content during a growing season, as well as other physiological and biochemical aspects of plants.

Infrared (IR) spectroscopy also showed good potential to be used as a prediction tool for total and individual FA content, as discussed in Chapter 8. Near-infrared reflectance (NIR) and Fourier-transform mid-infrared (FTMIR) spectroscopy both had similar accuracies meaning that either method could be developed further to the point where quantification of FAs may be possible. However, in order to achieve this, much larger datasets need to be used for developing accurate calibrations between FA data and spectroscopic data. Both methods have advantages and limitations in terms of their application, therefore the chosen method for development may depend on what long-term goal would like to be achieved. For example, FTMIR spectroscopy can be used on small quantities of plant material which is useful when working with limited numbers of plants. However, although NIR spectroscopy requires a large sample size compared to FTMIR, this may be beneficial when analysing more complex and inconstant forage samples such as those from mixed-sward pastures or silages.

In terms of the work done to try and enhance the understanding of the underlying biology and biochemistry of perennial ryegrass genotypes with differing FA content (Chapter 6 and Chapter 7), the main limitation here is with the number and nature of samples used to investigate the lipid composition of these genotypes in Chapter 7. Due to constraints on the amount of plant material remaining from the experiment discussed in Chapter 5, the investigation had to be limited to twenty-four genotype composite samples from one harvest. Akin to the limitations of the work discussed in Chapter 4, using material from one harvest limits the results to only being a snapshot of the plants

at the single moment in time. It would have been very interesting to have been able to investigate the seasonal effects in addition to the genotypic effects on lipid composition However, a number of factors prohibited this such as insufficient plant material and lengthy analytical methods.

9.3 Main Findings

Chapter 3

A positive relationship was found between *in vivo* chlorophyll, estimated using a *SPAD*-502 chlorophyll meter, and FA content. Additionally, the two varieties of perennial ryegrass used in this study which had been selectively bred for divergent *SPAD* also were found to be divergent in FA content. However, the relationship between *SPAD* and FA content was rather low.

Chapter 4

A strong positive relationship was found between FA content and CP whereas a negative relationship was found between FA content and WSC. However, this relationship was moderate due to the WSC content of the plants being somewhat variable.

Chapter 5

It was found that chlorophyll and FA content of leaves generally increases during a growing season. Also, significant differences between genotypes in terms of chlorophyll content, FA content and FA composition were observed; and these differences remained largely consist across the growing season. Positive relationships were found for *in vivo* chlorophyll and *in vitro* chlorophyll with FA content, however the *in vivo* chlorophyll relationships with FA content and *in vitro* chlorophyll content were relatively low and variable across the season.

Chapter 6

Content of individual and total FAs positively correlate across the growing season, with some correlations being more consistent between cuts than others. An interesting pattern was observed when correlating the proportions of individual FAs, with most correlating positively apart from with C16:1*trans*-3 and C18:3n-3. Some of these relationships can be explained by the FA and lipid biosynthesis pathways of plants while other remain unresolved.

Chapter 7

This work demonstrates that genotypes with increased TFA content have increased proportions of galactolipid, in addition to increased total chlorophyll content. The increased TFA content has minimal effect on the proportion of phospholipids and a negative effect on proportion of neutral lipids.

Chapter 8

Both NIR and FTMIR spectroscopy have good potential to be used to estimate FA content. The calibrations created in the current study are sufficient for screening purposes, with the exception of C18:1*cis*-9, however with the addition of larger datasets more accurate calibrations could be achieved which may lead to IR spectroscopy being used an alternative method to quantify FAs.

9.4 Implications

The work presented and discussed in this thesis complements previously published studies which have investigated seasonal and genotypic effects on FA content and composition of forages. However, the work here has specifically focussed on leaf FA content and composition, with the discovery that leaf FA content increases during a growing season. This work has also provided further evidence for the genetic aspect of FA content and composition, through finding significant differences between

genotypes within a population and that these difference generally remain consistent across the growing season. This implies that selectively breeding for increased FA content is possible regardless of environmental effects on this trait. However, other traits of interest such as CP and WSC, as well as other nutritionally and agronomically important traits, also need to be monitored as some of these traits have antagonistic relationships with FA content (as noted with FA and WSC content).

The work investigating the relationship between total chlorophyll and FA content also complements the limited number of publications which have previously assessed this relationship. In addition, the work presented in Chapter 5 has established how the relationships between both *in vivo* and *in vitro* chlorophyll content vary across a growing season. The strong and consistent relationships between *in vitro* chlorophyll and FA content show that chlorophyll has good potential to be used as a proxy for estimating FA content. However, development of more precise methods to determine chlorophyll *in vivo* are needed in order to accomplish good FA prediction.

Infra-red (IR) spectroscopy also holds great potential in the ability to predict FA content of plants. Near-infrared reflectance (NIR) spectroscopy is now commonly and commercially used to predict other nutritional characteristics of forages, particularly CP and WSC, so why not FA content also? Fourier-transform mid-infrared (FTMIR) spectroscopy is less developed compared to NIR, however it is becoming more frequently used within research due to advantages such as the need for a smaller sample size for this technique. Although the FA content of forages is quite low, calibrations with good accuracies and reasonably high linearity were achieved by both NIR and FTMIR spectroscopy for total and individual FAs, with the exception of C18:1*cis*-9. With the addition of larger data sets and further development of calibrations, both NIR and FTMIR have the potential to be able to quantitatively

predict FA content of forages. Infrared (IR) spectroscopy is much quicker, less destructive and relatively cheaper than the traditional wet chemistry methods of determining FAs via gas chromatography (GC). It terms of advantages in breeding for this trait, NIR or FTMIR could be used as a quick and inexpensive tool for screening plants for FA content; thus accelerating the selection and breeding process in addition to reducing the number of samples, amount of time and resources required for the wet chemistry analysis.

Most importantly, this work has also tried to uncover some of the basic biology and biochemistry surrounding the FA content and composition of perennial ryegrass. Establishing the relationships between the individual and total FAs has produced some interesting results, particularly with regard to proportions of FA. Some of these relationships have been explained by the FA and lipid biosynthesis pathways of plants while other remain unresolved. Further investigation into the biological and genetic control of FAs and lipids in forages may highlight control points which could potentially be exploited in order to increase the TFA content or alter the FA composition of forages. Investigating the lipid composition of a range of perennial ryegrass genotypes has also revealed that an increase in the proportion of galactolipids is accountable for increased TFA content. As previously mentioned, further work is required to determine whether this is a result of increased chloroplast size (due to increased number of thylakoid membranes) or whether it is a result of an increased number of chloroplasts. The effects this may have on overall plant health and performance also need to be considered, as well as the effects this may have in terms of lipid digestion in ruminants.

9.5 Further Research

There is a reasonable amount of published research which has investigated environmental effects on FAs in forages. A numbers of studies have also investigated the differences in FA content and composition between several species and varieties of forages, but very few have investigated FA differences at a genotypic level. Currently, little is known about the genetic control of FAs and lipids in forages, however, exploration of this is beginning to gain moment. Indeed, recent work carried out at Aberystwyth University has successfully identified quantitative trait loci (QTLs) associated with the major FAs found in perennial ryegrass (Hegarty *et al.*, 2013). This genetic research is being continued via the BBSRC funded 'Lipigrass' project (http://www.lipigrass.uk) in concordance with the expanding availability and accessibility of genomic based analyses. This project is also investigating high-throughput phenotyping methods and is developing the FTMIR work further through the use of larger FA datasets. Incorporating these techniques into a perennial ryegrass breeding programme will increase the efficiency and speed of the selection and breeding process, as it will reduce the requirement and costs associated with traditional phenotyping analyses such as GC analysis of FAs.

Aside from investigating genetics for knowledge and breeding purposes, other research groups are using genetic engineering to manipulate the FA and lipid composition of plants. Researchers in New Zealand are attempting to increase the energy density of forages by increasing TAG accumulation in leaves. This has been achieved through the co-expression of a synthetically modified structural protein, Cys-oleosin, and a diacylglycerol acyltransferase, DGAT1, in *Arabidopsis thaliana* and in perennial ryegrass (Winichayakul *et al.*, 2008, 2013). There have also been recent publications from the USA, France and China investigating the genetic and

transcriptomic regulation of FAs and lipids in plants (Fan *et al.*, 2013; Marchive *et al.*, 2014; Wang *et al.*, 2015). Additionally, work being carried out at Rothamsted in the UK is focussing on creating genetically modified Camelina (*Camelina sativa*) to produce LC-PUFA such as EPA and DHA (Napier *et al.*, 2014).

Investigation into the lipid composition of forages is much more limited, with the majority of publications dating back to the 1960s and 1970s. Gaining better knowledge and understanding of lipid biochemistry in plants is an essential part of understanding FAs in plants. However, a great deal more research is needed to investigate and establish environmental effects on lipid composition of forages in addition to genetic influences and control.

9.6 General Conclusions

To conclude, the aims and objectives set out at the beginning of this thesis have been accomplished. The seasonal variation and genotypic differences in FA content and composition of perennial ryegrass has been investigated. Genotypic effects on lipid composition have also been investigated, however investigation of seasonal effects was prohibited. The relationships between FAs and other nutritionally important traits (CP and WSC) have been explored and discussed, as well as the relationships between FAs themselves. Also, FA prediction tools based on chlorophyll content and infra-red spectroscopy have been evaluated.

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